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THE EFFECT OF AIR-GENERATED SOUND WAVES ON SUSPENSIONS OF MICROORGANISMS

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Wright Air Development Center
Air Research and Development Command
United States Air Force
Wright-Patterson Air Force Base, Ohio

FOREWORD

This is a report of the work done by the bacteriology section of The Pennsylvania State College research project on the biological effects of high intensity sound. The report includes experimental work done from the inception of the project in the spring of 1949, through the time when writing was started in Oct. 1951. The work has been generously supported by the U.S.A.F. under Contract AF 33(038)-786, RDO No. R-695-63 "Effects of Vibration on AF Personnel."

This work was written up at this time at the specific request of Major H. C. Farrack, project engineer. The writing was done by the senior bacteriology and physics personnel, but the actual experimental work has been carried out by a large number of people including the following:

James J. Reid, Professor of Bacteriology
Helen L. Kinsloe, Instructor of Bacteriology
Alan I. Gutowitz, Graduate Student in Bacteriology
Bohdan R. Choman, Graduate Student in Bacteriology
Andrew J. Law, Graduate Student in Bacteriology
Jesse W. Tieman, Graduate Student in Bacteriology
Catherine F. Gray, Medical Technologist

Wesley L. Nyborg, Assistant Professor of Physics
Robert J. Nertney, Graduate Student in Physics
Joseph W. Spencer, Graduate Student in Physics
Ralph Simon, Assistant Professor of Physics
Eugene Ackerman, Assistant Professor of Physics
Frederick E. Bellas, Graduate Student in Physics
William Holak, Graduate Student in Physics

For the first two years this project was under the direction of Harold F. Schilling, Professor of Physics and Dean of the Graduate School, and profited from his guidance. It is currently under the direction of Michael A. Farrell, acting Director of the Agriculture Experiment Station and acting Vice-Dean of the School of Agriculture.

ABSTRACT

Two high intensity air borne sound generators, a siren and a jer T type whistle, were used to study the effects of sound on liquid suspensions of bacteria. Lethal effects and cellular disruption were observed when the suspensions were exposed in specially constructed exposure chambers. These effects were most pronounced with certain strains of Micrococcus pyogenes var. aureus but were irreproducible over a period of months.

The experiments failed to elucidate the mode of action of the acoustic field in producing lethal effects. The data do show that bacteria can be killed in standing wave fields, with maximum pressure amplitudes of a few tenths of an atmosphere and maximum particle velocities of a few centimeters. The threshold for these effects appears to be very sharp.

It is concluded that although these sound sources are impractical for enzyme or antigen extractions, they do indicate a relative scale of bacterial sensitivity to these sound fields.

PUBLICATION REVIEW

This report has been reviewed and is approved.

FOR THE COMMANDING GENERAL:

Robert H Blount

ROBERT H. BLOUNT
Colonel, USAF (MC)
Chief, Aero Medical Laboratory
Research Division

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SECTION I

INTRODUCTION

The lethal effects of high intensity acoustic fields acting on microorganisms suspended in a liquid medium have been known for sometime. An excellent review is presented by Gregg in Vol. II of Medical Physics (3) so that no comprehensive review will be attempted here.

The work to be described was initiated for several reasons. The most important of these were the following two:

1) Most past work on biological effects of sound in liquid has been carried out in poorly defined acoustic fields. Often the DC voltage applied to the plate of the electronic oscillator was the only recorded indication of acoustic field strength. This makes the comparison of work by different people using different equipment almost impossible. It was felt that testing procedures and instrumentation had progressed at The Pennsylvania State College to the point that acoustic fields could be precisely described. (This feeling was in part based on experiments in air borne sound and a failure to realize the complications of liquid media.) To work with the physicists there were on the campus qualified and interested bacteriologists. Thus the personnel and equipment appeared ideal for a project of this nature.

2) It was desired to build apparatus for sonic disintegration of bacteria which would be cheap, easy to construct and yet reliable in its performance. It was hoped that the T type jet whistle would meet this requirement. Again whistles and adequate physics and bacteriology personnel were all on campus.

It is our hope in writing this report that all of the sections can be read with understanding by either a physicist or a bacteriologist. The numerous limitations which make our results hard to interpret will be discussed. In particular we will indicate why air borne sound sources are not very useful for bacteriology experiments.

The experiments described in this report depend on a) special bacteriological techniques and b) a source of sound in air which is then coupled to the liquid. The two sound sources employed were a whistle and a siren. When using the siren special exposure chambers had to be constructed which we describe. Brief descriptions are given of both the whistle and the siren, to make their physical forms and pertinent acoustic properties clearer to the reader.

In general physical agents may exhibit four principal effects

on microorganisms, namely: stimulation, attenuation, variation and killing. The possibility of demonstrating one or more of these effects was considered in choosing the species to be used as test microorganisms. Also in choosing the microorganisms general group characteristics were considered. There are representatives of the Gram positive non spore forming rods, one spore forming species, Gram negative rods and Gram positive cocci. Some of the species are strictly aerobic while others are microaerophilic or facultative in their oxygen requirements. Resistance to other physical agents such as heat and ultraviolet light and optimum growth temperature were also considered. There are pigmented and non pigmented species, hemolytic and non hemolytic, as well as some that have been studied extensively in regard to serological characteristics.

Among the Gram positive cocci Micrococcus pyogenes var. aureus represents a truly spherical cell and produces a characteristic orange pigment. It is a standard test microorganism used by the Federal Drug Administration for the evaluation of disinfectants and is used for the testing of antibiotics. Also any change in the ability of pathogenic strains of this species to produce coagulase or hemolyze erythrocytes would be easily detected. Micrococcus varians was chosen because of its thermoduric property. Sarcina lutea represents a somewhat larger spherical cell than any of the other cocci included in this study, the cells tend to clump together very tightly in packets, it produces a yellow pigment and is quite resistant to desiccation and ultraviolet light as shown by its presence in air. The two Streptococcus species represent cells that tend to be slightly ovoid rather than truly spherical and tend to form chains rather than clusters or packets. Streptococcus zymogenes will grow at a higher temperature than Streptococcus lactis. The two species are markedly different serologically as Streptococcus zymogenes is a perfect example of the Lancefield Group D hemolytic streptococci and Streptococcus lactis is serologically more like the viridans group and Diplococcus pneumoniae and is non hemolytic. Both species are microaerophilic and do not produce catalase.

Of the Gram positive rods Lactobacillus casei, Corynebacterium michiganense and Corynebacterium xerose were chosen because they are non spore forming rods and do not exhibit marked resistance to physical agents. Lactobacillus casei is acid tolerant and microaerophilic. Corynebacterium xerose is a human parasite while Corynebacterium michiganense is a plant pathogen. Bacillus megatherium var. danicus was chosen because it is a relatively large spore forming rod and is strictly aerobic.

Of the Gram negative rods Pseudomonas aeruginosa was chosen because its ability to grow on simple synthetic media would make it useful in possible nutritional or other variation studies. Also, it produces a pigment that diffuses into the medium while

the other pigmented forms do not produce this type of pigment. It is aerobic and hemolytic. Aerobacter aerogenes and Escherichia coli are facultative intestinal organisms that are used as an index of pollution in the bacteriological analysis of water. Aerobacter aerogenes forms relatively large mucoid colonies because of the production of capsular material. Escherichia coli has been used by several other workers investigating the effects of sound waves. Also there are a number of well studied variant strains available. Serratia marcescens was included because its pigment apparently helps to protect it from the destructive effects of ultraviolet light. Salmonella typhosa has been extensively studied serologically and also is used by the Federal Drug Administration as a test microorganism for the evaluation of disinfectants.

The yeast, Saccharomyces cerevisiae, was included because it is larger and has a more organized structure than a bacterial cell and has been the subject of much physiological study. Also its method of reproduction differs from that of bacteria.

SECTION II

MICROORGANISMS AND MICROBIOLOGICAL TECHNIQUES

STRAINS OF MICROORGANISMS EMPLOYED

The following 17 strains of microorganisms were used in this investigation.

Micrococcus pyogenes var. aureus. Three strains of this organism were used and have been designated as Strains I, II and III. Strain I is the F D A strain #209. It is a Gram positive coccus occurring in irregular clusters. Mannitol is fermented without the formation of gas. An acid curd is produced slowly in litmus milk, gelatin is liquified slightly and nitrates are reduced to nitrites. This strain is non hemolytic and does not produce coagulase. Strains II and III were isolated from human infections. They are culturally and morphologically similar to Strain I except that they are somewhat more active on milk and gelatin, beta hemolytic and produce coagulase. All strains produce an orange-yellow pigment on nutrient agar.

Micrococcus varians. The strain of this organism employed was isolated from pasteurized milk in the Department of Bacteriology of The Pennsylvania State College. The strain may be characterized as follows: the cells are Gram positive cocci occurring in pairs, short chains and clusters. A light yellow pigment is

produced on nutrient agar. Litmus milk is turned acid in about one week. Lactose, sucrose and glycerol are fermented with the formation of acid but no gas. Mannitol is not fermented and usually becomes alkaline. Gelatin is not attacked and the reduction of nitrates to nitrites is variable. At various intervals throughout the investigation, the thermoduric property of this culture was checked. A freshly inoculated broth culture was subjected to pasteurization temperature of 62.5°C for 30 min and then plated to determine the number of viable cells. It was found that, in every case, the counts of the treated cells compared favorably with the untreated controls.

Sarcina lutea. Strain #65 in the culture collection of the Bacteriology Department of The Pennsylvania State College was used for this study. It is a Gram positive coccus, the cells frequently occurring in packets of eight. Common sugars are not fermented; nitrates are reduced to nitrites; hydrogen sulfide is formed; indole production is slight and litmus milk is coagulated, becoming alkaline in 2-3 days. A lemon yellow pigment is produced.

Streptococcus lactis. The strain of this species used was isolated in our laboratory from a buttermilk starter culture and exhibits morphological and cultural characteristics typical of the species.

Streptococcus zymogenes. The strain of this organism used was originally obtained from Dr. Rebecca Lancefield, designated as C-1, and is one of the strains used by her in establishing Group D of the hemolytic streptococci. The strain was observed to conform to the description of Lancefield (5) except that gelatin is not liquified.

Lactobacillus casei. Strain #102 in the culture collection of The Pennsylvania State College was used. This strain was originally obtained from the University of Wisconsin. This strain is not quite as active on carbohydrates as typical members of the species.

Corynebacterium xerose. This strain was obtained from the New York State Department of Health, Division of Laboratories and Research, as strain #39165 and is typical of the species.

Bacillus megatherium var. danicus. Strain #36 of The Pennsylvania State College culture collection was used. It is typical of this variety.

Pseudomonas aeruginosa. The strain of this species used was #205 from the culture collection of The Pennsylvania State College and is typical of the species.

Aerobacter aerogenes. A soil strain, #174 in the culture collection of The Pennsylvania State College, was employed in this study.

This is typical of the motile strains of this species.

Escherichia coli. Strain #169 of the culture collection of The Pennsylvania State College was used. It is a motile strain that does not attack sucrose, raffinose, salicin and dulcitol.

Serratia marcescens. The American Museum strain, #185 in The Pennsylvania State College culture collection, was used. This strain is typical of the species.

In order to obtain a culture that would consistently produce about 75% pigmented colonies the following procedure was followed. The culture was plated on nutrient agar using loop dilutions in series and from the colonies appearing a perfectly smooth well pigmented one was selected for isolation. This process was repeated seven times.

Salmonella typhosa. The Hopkins strain, #116 in the culture collection of The Pennsylvania State College and #6539 in the American Type Culture Collection, was employed. This is the strain used by the Federal Drug Administration as a test organism in the evaluation of antiseptics by the Phenol coefficient method and is typical of the species.

Saccharomyces cerevisiae. This typical strain of baker's yeast is #257 in the culture collection of The Pennsylvania State College.

All strains were tested at regular intervals to assure that no change occurred in the morphological or physiological characteristics.

Media and incubation temperatures used for the cultivation of these microorganisms were chosen as suitable to the particular strain.

MEDIA AND INCUBATION TEMPERATURES

Standard Difco nutrient agar was used for the stock cultures and for plating the following species: Micrococcus pyogenes var. aureus, Micrococcus varians, Sarcina lutea, Bacillus megatherium var. danicus, Pseudomonas aeruginosa, Escherichia coli, Serratia marcescens and Salmonella typhosa.

Difco Tomato Juice Agar was used to carry the stock cultures and also for plating of Streptococcus lactis and Lactobacillus casei.

Four additional gm of agar were added to each liter of the medium to make it suitable for plating purposes.

Because of the microaerophilic nature of Lactobacillus casei

after the inoculated plates had hardened a layer of uninoculated tomato juice agar was poured into the plates to act as a seal.

Streptococcus zymogenes was cultivated on Difco Stock Culture Agar as preliminary studies showed this substrate to be superior to nutrient agar.

An additional 7.5 gm of agar were added to each liter of medium to make it suitable for plating.

Difco Tryptose Phosphate Agar was used for the cultivation of Corynebacterium michiganense and Corynebacterium xerose. The medium was prepared by adding 15 gm of agar to a liter of Difco tryptose phosphate broth.

Difco Wort Agar was used for the cultivation of Saccharomyces cerevisiae.

The following cultures were incubated at 37°C: Micrococcus pyogenes var. aureus, Micrococcus varians, Streptococcus zymogenes, Corynebacterium michiganense, Corynebacterium xerose, Bacillus megatherium var. danicus, Pseudomonas aeruginosa, Aerobacter aerogenes, Escherichia coli and Salmonella typhosa.

The incubation temperature used for the following cultures was 31°C: Streptococcus lactis, Lactobacillus casei and Saccharomyces cerevisiae.

Sarcina lutea and Serratia marcescens were incubated at room temperature; however when non-pigmented cultures of Serratia marcescens were desired the incubation was done at 37°C.

STUDIES IN THE PREPARATION OF BACTERIOLOGICAL SUSPENSIONS

On the basis of the report of Hamre (4) and other investigators it was assumed that the initial concentration of the suspension had little if any bearing on the effect of the sound wave. Extremely low concentrations were thought inadvisable because if death declines were found to be logarithimic, the errors due to plating would obscure the slopes. Extremely high concentrations, on the other hand, would be impractical, requiring extensive plating operations with corresponding materials and media and such suspensions might interfere with the action of the sound field (3). Initial concentrations falling between 300 thousand and 2 billion were finally chosen for the suspensions.

An investigation was made to find a suspending medium that would not effect the viability of the cells. Comparison was made of unbuffered saline (0.85% NaCl), a buffer solution consisting of a 1:2 mixture of a 1% solution of monosodium phosphate and a 10% solution of disodium phosphate (pH 7.0) and a combination

of the buffer solution and physiological saline. Unbuffered physiological saline was found to be as satisfactory bacteriologically as the other solutions and was used in most of the exposures.

The possibility that the organisms might increase or decrease in numbers in the suspending medium was examined and it was found that the organisms may be held in physiological saline in the refrigerator at least three days without appreciable change in numbers.

In cognizance of the possibility that the growth phase might prove a factor in relative resistance to sonic waves, the growth curves for Micrococcus pyogenes var. aureus and Serratia marcescens were determined thus making it possible to state the growth phase of the organism at any exposure.

Preliminary studies indicated that great care must be taken in the preparation of a suspension to avoid any apparent increase in viable cells following short sonic exposure. Repeated tests indicated that this apparent rise could not be accounted for on the premise of stimulation of cells to form colonies that would in the absence of sonic waves fail to reproduce. On the other hand the apparent rise in numbers could be accounted for on the basis that the sound waves broke up chains and clumps of cells that had not been broken up by the agitation in the suspending process. Accordingly the following technique was developed which for most strains used resulted in suspensions which did not show a great rise in numbers following brief sonic irradiation. Cultures for suspensions were made by transferring with a needle from an agar stock culture to slants, of a medium suitable for the strain in question. After suitable incubation, again depending on the strain in question, each slant was washed with 2 milliliters of sterile physiological saline. Care was taken not to dislodge any of the nutrient material.

The suspended cells from two slants were then placed in 9 milliliters of sterile physiological saline in a test tube, making a total volume of approximately 13 milliliters. This suspension was shaken by hand for about 3 min. The hand shaking broke up gross clumps and resulted in a smooth suspension corresponding to tubes #2 to #4 of a Barium Sulfate Nephelometer Standard. Approximately 2 milliliters of this hand shaken suspension were then transferred to 80 milliliters of sterile physiological saline contained in a 500 milliliter Erlenmeyer flask to form the final suspension. To further insure homogeneity, the flask was shaken for 30 to 60 min on a Fisher-Kahn shaker.

Where broth suspensions were desired, 50 milliliters of nutrient broth, contained in a 500 milliliter Erlenmeyer flask, were inoculated with 1 milliliter of a 24 hr broth culture. Just prior

to exposure, the flask was shaken on a Fisher-Kahn mechanical shaker for at least 30 min.

DETERMINATION OF SONIC EFFECTS

To determine sonic effects plate counts, morphological, physiological and serological techniques were used. Counts of viable cells were made by the plate method as recommended by the American Public Health Association in the Ninth Edition of Standard Methods for the Examination of Dairy Products with the exception of medium employed and temperature of incubation. Serial dilutions from 1:100 to 1:10,000,000 were made with the use of sterile 99 milliliter dilution blanks and 1.1 milliliter milk pipettes and duplicate plates were poured for all dilutions. The medium employed and the temperature of incubation for each strain has been described previously.

Incubation periods were usually 48 hr but at times the plates were re-examined at 72 hr. Colony counts were made with the aid of a Quebec Colony Counter, and the rules of the American Public Health Association with regard to counting were observed.

To determine morphological characteristics smears were made of the exposed suspensions and were stained by the Gram technique. Photomicrographs of stained preparations of Micrococcus pyogenes var. aureus were made by the use of a Leitz "Micro-Ibso" camera attached to a light microscope.

To determine physiological characteristics suspensions of cells exposed to the sound field of the siren and progeny of exposed cells were subcultured in various media to detect possible changes in the characteristic physiological activity of each species of microorganism used.

In the case of Micrococcus pyogenes var. aureus exposed cells, progeny of exposed cells, cell-free filtrates from both exposed and unexposed suspensions were tested for hemolytic and coagulase activity. Bovine blood agar was streaked and incubated at 37°C for 48 hr to determine hemolytic activity. Coagulase activity was determined according to the procedure of Chapman et al (2). In this technique 0.5 milliliter of citrated human blood is mixed with 0.5 milliliter of the sample to be tested and the mixture incubated at 37°C for a period of 3 hr. Coagulation within the 3 hr period is considered a positive test for coagulase.

DETERMINATION OF THE RELEASE OF ANTIGENIC SUBSTANCES FROM EXPOSED CELLS

In all serological studies of filtrates of suspensions exposed to the siren Micrococcus pyogens var. aureus, Strain II, was employed. Accordingly this strain was used in the production of

antisera for the serological tests.

Preparation of Vaccine. A nutrient broth culture incubated at 37°C for 24 hr was used in the inoculation of nutrient agar bottle slants. Each slant received 0.5 milliliter of the broth culture and was incubated in a horizontal position at 37°C for 24 hr with periodic tilting of the bottle to obtain uniform growth. At the end of the incubation period, 5 milliliters of 0.3% formalized physiological saline were introduced into each bottle and the growth placed in suspension by moderate shaking. The suspended cells from six bottles were pooled in a 50 milliliter test tube containing 10 glass beads. This tube was placed in the Arnold at 100°C for 30 min. Sterile cotton was then pushed through the suspension to get rid of large clumps of cells. Next the suspension was diluted with 0.3% formalized saline to equal tube #2 of the Barium Sulfate Nephelometer Standard. Following this dilution the suspension was placed in a sterile 50 milliliter vaccine bottle closed by a rubber stopper. The vaccine was tested for sterility by culturing in thioglycollate broth. When not in use the vaccine was refrigerated.

Immunization of Animals. The vaccine was placed into the posterior auricular veins of healthy 5 lb male albino rabbits by means of a 24 gauge, three-quarter in. needle. The inoculation schedule of the rabbits with the vaccine was as follows:

Inoculation	Days after first inoculation	Inoculum in milliliters
1	-	0.5
2	2	1.0
3	4	2.0
4	6	3.0
5	8	4.0
6	10	5.0

The rabbits were allowed to fast for a 24 hr period before bleeding in order to obtain clear serum. Beginning five days after the last inoculation, the animals were bled from the heart on three consecutive days. Approximately 30 milliliters of blood were drawn each time. The serum was obtained by rimming the clot after the blood had stood at room temperature for 1 hr, pipetting off and centrifuging at 30,000 rpm for 15 min. The sera obtained from five animals were then pooled and tested for titer.

Determination of Antisera Titers by the Agglutination Test. The standard tube method was employed in this test. The growth from the surface of a 24 hr nutrient agar slant culture was washed with 10 milliliters of 0.85% saline. The suspension was homogenized with the aid of a pipette filtered through cotton to

remove any clumps of cells and placed in a clean dry test tube. The filtered suspension was diluted with 0.85% saline until it corresponded in turbidity with tube #2 of the Barium Sulfate Nephelometer Standard. This suspension was then used as the testing antigen.

A series of 10 seriological tubes was arranged to contain dilutions of antisera ranging from 1:10 to 1:2560; 0.5 milliliter of the respective dilution was placed in a tube. To each tube, 0.5 milliliter of testing antigen was added and the contents were well mixed by shaking. Sensitization was accomplished by holding the tubes in a water bath at 56°C for 2 hr followed by overnight storage at 10°C. The agglutination titer was then recorded. Pooled antisera made and tested in this manner showed a titer of 1:1280.

Determination of Antisera Titers by the Precipitin Test. A testing antigen prepared by acid extraction of untreated cells was used at regular intervals to check the precipitin titers of the antisera produced. If the titer of a stored antiserum decreased significantly, it was discarded and a fresh antiserum was used. In the experimental work with sound treated suspensions the titer obtained with the acid extracted antigen was routinely compared with that obtained from the sound extracted antigen. The acid extracted antigen was prepared according to the technique of Lancefield (6) for use with Streptococcus.

Examination of Treated Material by the Precipitin Test. Exposed samples were filtered through a #3 Seitz filter to obtain a cell-free filtrate for serological study. This filtrate was used in dilutions ranging from 1:1 to 1:16,384 in the precipitin test. Using Pasteur pipettes, 0.1 milliliter portions of the series of filtrate dilutions were layered over 0.1 milliliter portions of undiluted antiserum in a series of 15 serological tubes 6 x 50 mm in size. In addition to the filtrates from exposed suspensions, filtrates from unexposed suspensions were layered over antiserum. Also the exposed and unexposed filtrates were tested against normal rabbit serum. Sensitization was accomplished at room temperature in a period of 1 hr. The appearance of a white ring at the junction of two layers constituted a positive test.

SECTION III

SIREN EXPERIMENTS

THE SIREN

The source of sound illustrated in Figs. 1, 2 and 3 used in these experiments is an air-driven siren designed by Allen and Rudnick (1). When it is in operation, compressed air is released in bursts upward from the top face of a metal chamber. This air emerges from the chamber into the open through 100 small vertical holes drilled in the top face, spaced at equal intervals on a circle 6 in. in diameter. These holes are alternately opened and closed simultaneously by 100 teeth spaced evenly on the periphery of a whirling stainless steel rotor which fits snugly against the top face of the chamber. A sound field is generated by the resulting regular series of air bursts; its fundamental frequency is equal numerically to the number of teeth cutting past a given outlet port per sec and with this the siren can be varied from 4 to 34 kc. In general the spectrum at any point in the field contains many higher harmonics in addition to the fundamental. Although the intensity of these harmonics was very large at some places no attempt was made to evaluate their possible effects on the biological results. We have records of only one set of measurements which was made to compare the total intensities with the fundamental intensities. On the basis of this single experiment made in the absence of the bacterial exposure chamber, it was decided that the harmonics made a very small contribution to the problem. No other measurements were made of anything but the fundamental. In reporting these experiments we realize that this disastrous gap in the data makes a reasonable interpretation little more than guess work.

When a sound field exists in a fluid such as air or water, it implies that there are cyclic variations in time of the density, pressure, temperature and velocity of portions of the fluid. Variations of these quantities are by no means always proportional; thus at some points in a field the pressure variations may be large while those of the velocity are small, or the reverse may be true. Measurements will be reported here of the sound pressure in regions of interest; the instantaneous value of this quantity is defined as the difference (positive or negative) between the actual pressure in some region at a given instant and the time-averaged value of the pressure in that region. If several harmonics are present, the pressure amplitude may be determined separately for each. As mentioned before, it is recognized that a sound field is not completely specified by knowledge of the sound pressure distribution; this quantity is selected for consideration since it can be measured conveniently. However it is not at all clear that the sound pressure is the significant variable to measure. It would be desirable to have data on at least one other

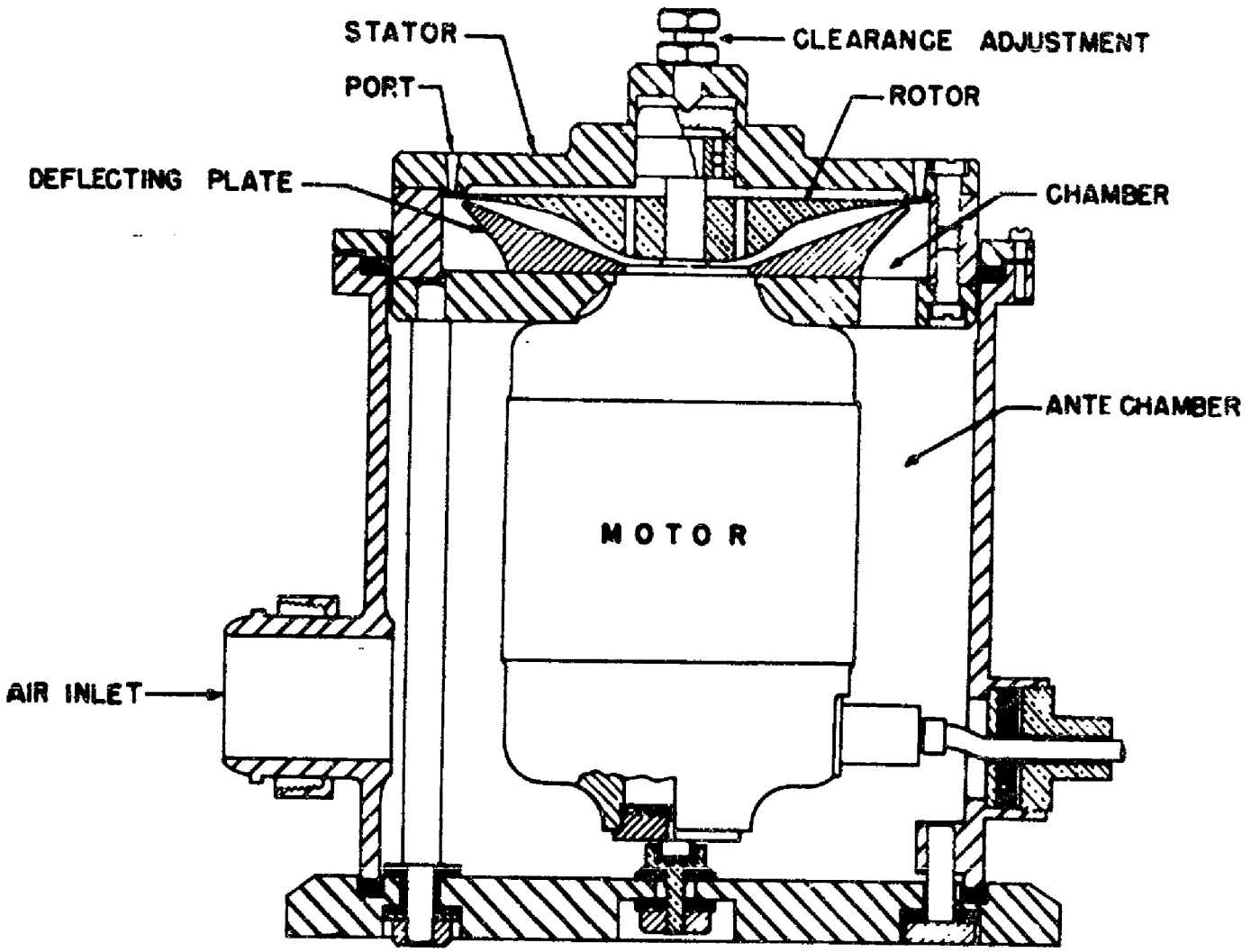


Fig. 1 Cross section of siren.

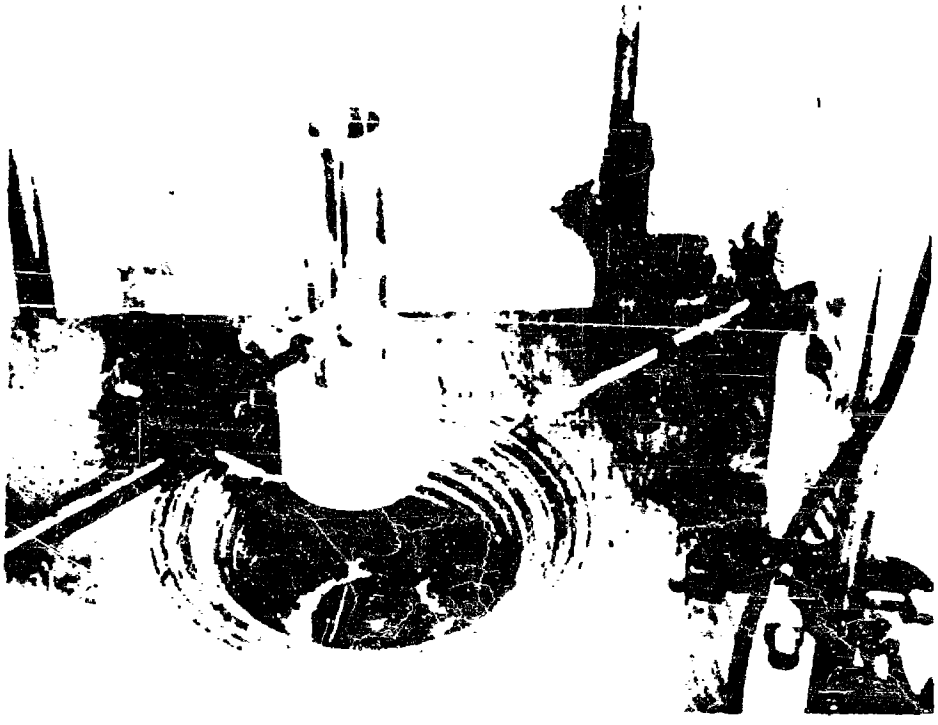


Fig. 2 Exposure container in position above siren.

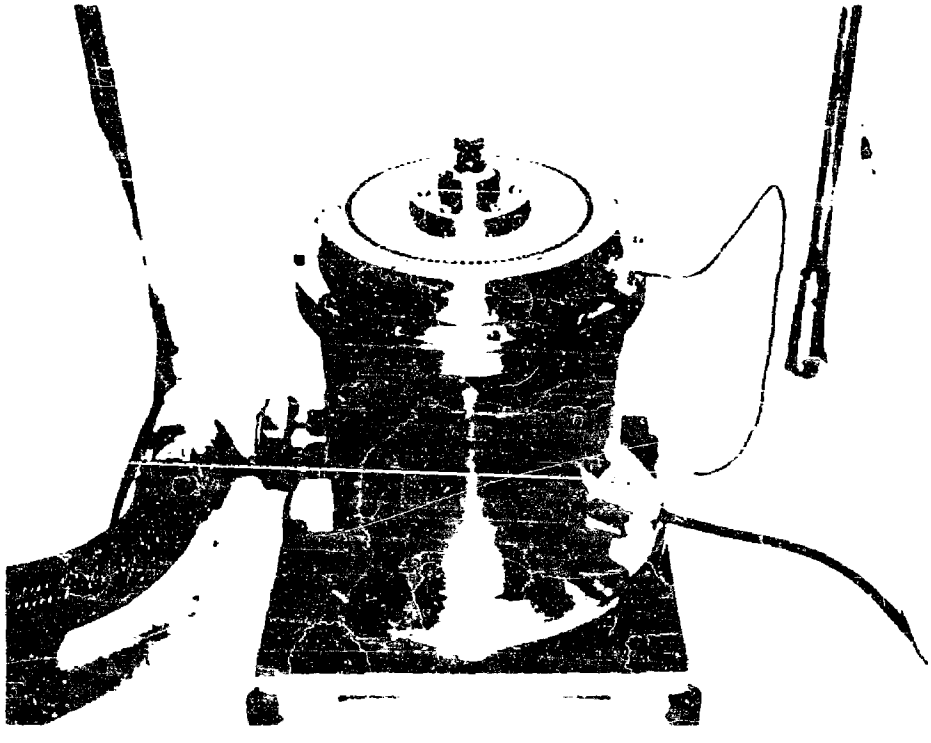


Fig. 3 Siren.

variable such as the particle velocity which is not simply related to the sound pressures in the complex acoustic fields present in the exposure chambers used. However sound pressure measuring devices were the only type available to the experimenters when the research was carried out.

The measurements of sound pressures were accomplished by use of a ceramic probe microphone. When the tiny sensitive element of this unit is placed in a selected region of a sound field, it generates an alternating voltage whose frequency is that of the sound and whose magnitude is proportional to the sound pressure in the region of interest. The frequency and magnitude of this voltage are measured with an electronic analyzing system. Frequencies of the sound are determined by such an arrangement to within about $\pm$ 3% maximum error. On the other hand, the absolute measurements of sound pressures in air are subject to about $\pm$ 20% probable error, although comparison of two different sound pressures may be made with less than 5% maximum error.

As used in the work reported here, the siren is mounted so that the axis of its rotor is vertical. For a given frequency the sound pressure at any point in the field above the source depends on the air flow to the siren; the sound pressure amplitude always increases, within limits, as more air is forced through the generator. With both frequency and airflow fixed the point of maximum pressure amplitude is found to be near the siren and on its axis; here the fundamental component may be as much as 0.1 at. pressure amplitude under favorable conditions. The sound pressure at points along the axis falls off steadily with height above the sound source. Also it is found to decrease if one proceeds radially out from the axis at any given height; the rate of the latter decrease is dependent on frequency, being greater at higher frequencies. The region immediately near the axis is referred to as the central lobe of the siren; speaking very roughly the siren concentrates the greater part of its sound energy in a beam along this central lobe.

Exposures with the container, shown in Fig. 11, were made by placing the latter vertically in the central lobe, centered on the axis, with the Koroseal diaphragm 15 cm above the siren's face. At all frequencies considered here the pressure amplitude for an airflow corresponding to maximum siren output on the axis at a height of 15 cm is about 0.08 at. or 168 db above the threshold,* before the exposure container is placed there; this figure will be referred to as the free field value of the sound pressure on the axis at 15 cm for the conditions considered.

After the exposure container is placed in position the sound

* The number N of db above threshold corresponding to a pressure amplitude p is given by:

$$N = 20 \log_{10}(p/0.00028),$$

where p is the pressure amplitude in dyne cm^{-2}

pressure is, in general, considerably modified from its free field value. The free field pressure amplitude is not constant over the area to be occupied by the lower end of the container. The greatest free field sound pressure occurs at the axis; at points 2.5 cm (the radius of the exposure container) off the axis, the free field sound pressure may be down by a factor of several db.

Measurements were made of pressure amplitudes for the fundamental frequency as they existed in liquid samples under actual exposure conditions. In so doing the bacterial suspension was replaced by an equal volume (30 milliliters) of degassed water. Readings were found to be erratic if air was present. Results for 6.4, 10.0, 15.0 and 24.5 kc are presented in Figs. 4 and 5. These measurements are subject to an error of almost $\pm$ 30%. In Fig. 4 for 6.4 kc, as in the following graphs, it is shown how pressure amplitudes vary with height above the Koroseal diaphragm; the upper curve is for points along the axis while data for the lower one were obtained at points just inside the stainless steel wall of the container. Heights are in cm while pressure amplitude is plotted in terms of db above the free field value corresponding to a point at the center of the Koroseal diaphragm. To obtain actual pressure levels in db above threshold from the values given in Fig. 4 and in the other similar graphs in Fig. 5, simply add to them the appropriate free field value. For example, at 6.4 kc the greatest free field level used was 168 db above threshold. In this case the greatest pressure amplitude in the container, that which exists on the axis at the bottom, is 5 + 168 i.e. 173 db above threshold; this is equivalent to about 0.13 at.

It should be noted that at any given height pressure amplitude decreases as one proceeds horizontally from the axis outward to the wall. As may be seen in Fig. 4 the amount of decrease is greatest at the bottom of the container where a difference of nearly 5 db exists over a horizontal section. Toward the top of the liquid, at 1.5 cm, the horizontal sound pressure distribution is more nearly uniform. Thus at 1.2 cm, pressure amplitude is only 2 db greater on the axis than at the wall; at the same time, though, both pressures are much less at 1.2 cm than at the bottom of the container.

Fig. 5 shows similar measurements of pressure amplitude (for the fundamental) at frequencies of 10.0, 15.0 and 24.5 kc. A wide variety of sound pressure distributions is indicated in these values. Measurements of a similar kind at 5.0 kc were not completed; at the bottom the pressure amplitudes on the axis and at the wall are, respectively, 6 db and 3 db above the free field value for this frequency.

To properly evaluate the bacterial data to be presented certain difficulties in the physical measurements should be

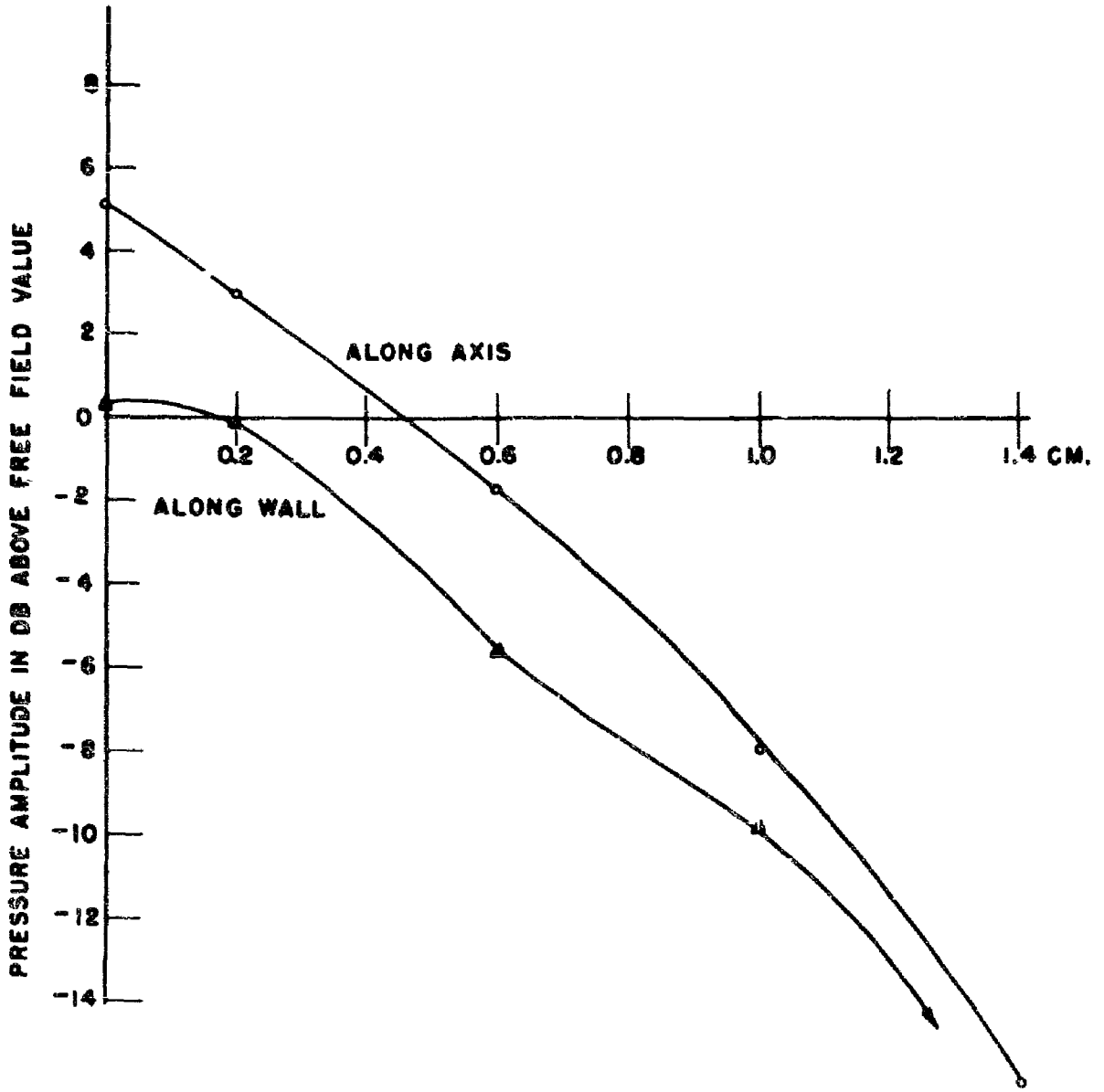


Fig. 4 Pressure amplitude versus height above diaphragm for exposure container shown in Fig. 11; frequency 6.4 kc; free field intensity 168.5 db above threshold at center of diaphragm.

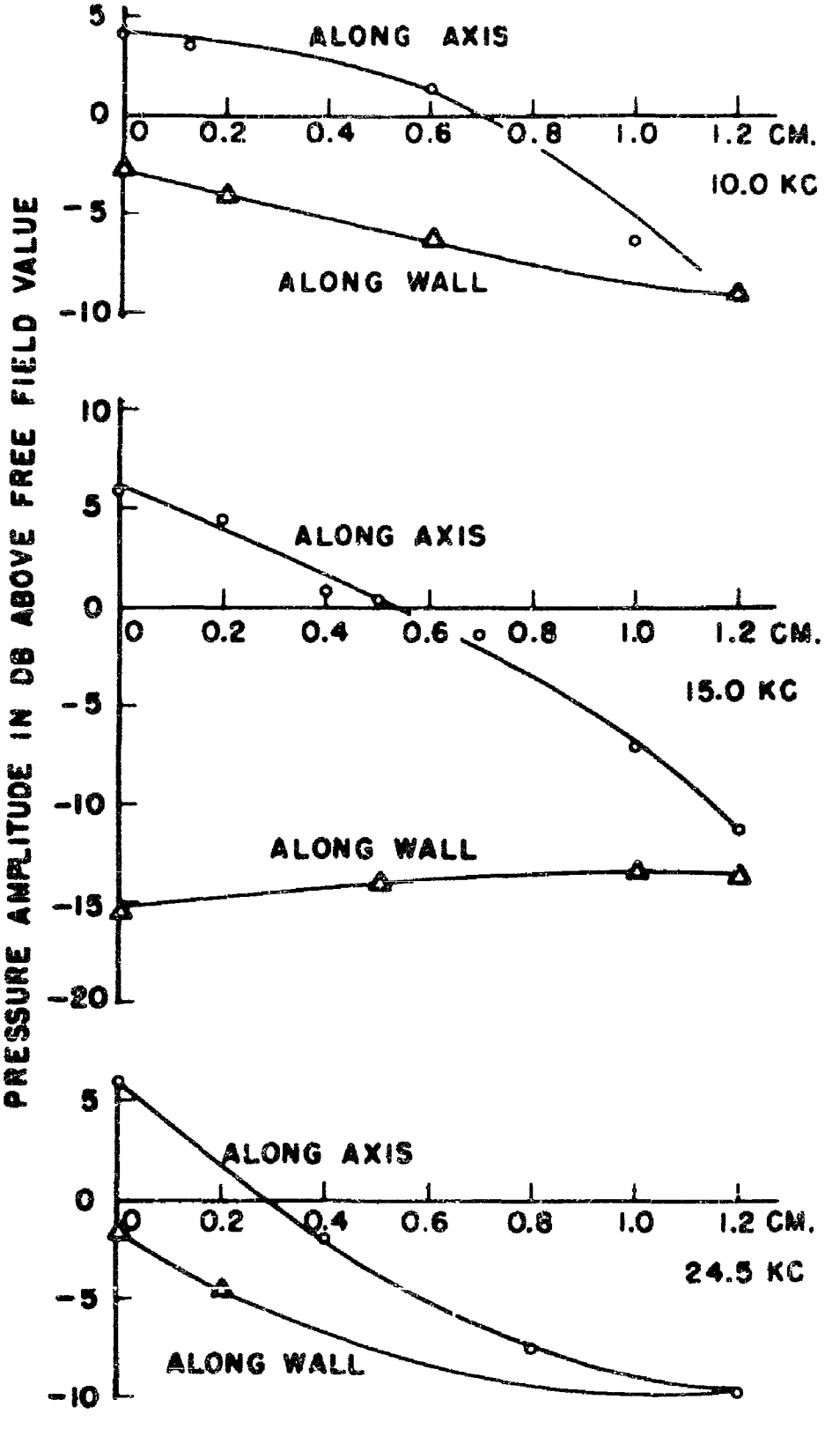


Fig. 5 Pressure amplitude versus height above diaphragm for exposure container shown in Fig. 11; frequencies 10.0, 15.0 and 24.5 kc; free field intensity 168.5 db above threshold at center of diaphragm.

appreciated. In the first place it was usually impossible to keep the output of the siren constant; over the course of an hour this often changed as much as 3 db. No records are available to indicate that these changes were either measured or compensated for in any way. A second difficulty was that a very slight tilt in the bacterial exposure chamber radically altered the acoustic field in the liquid. Since the probes could not be sterilized, it was impossible to monitor in any way the acoustic field in the liquid. The third and last difficulty was the failure to monitor the harmonic content. There is no reason to believe that the harmonic content in the liquid was the same as the free field harmonic content at the location of the base of the exposure chamber. Nor do we have any data indicating the consistency or insignificance of the harmonics except on one occasion which might have been atypical. Recent measurements indicate a tremendous day to day variation. The siren in its present condition is sufficiently changed to make meaningless any further purely physical checks. Thus we present the siren data for its historical interest as a background to the whistle experiments, and as an indication of difficulties which arise in this type of experimental work.

DEVELOPMENT OF EXPOSURE CONTAINERS

The development of exposure containers suitable from the standpoints of the bacteriologist and the physicist posed the initial problem of this investigation. The problem of a container for use with the siren was first considered.

Preliminary experiments showed that laboratory glassware was not suitable as the glass was apt to shatter when placed in the sound field. The first metallic container built for this study is illustrated in Fig. 6. It consisted of a stainless steel ring to which a 10 in. steel rod was attached. The volume of the container was 2 milliliters. The top of the container as well as the bottom had to be covered with a diaphragm to avoid spraying of the bacterial suspension. For this purpose three materials were tested, sheet rubber, scotch tape, and a plastic known as planpak. Various methods of gluing and taping these diaphragms to the container were attempted but none proved entirely satisfactory. The ring was discarded because: it was difficult to prevent leakage; it could not be autoclaved; the volume was smaller than was desired; and the contents could not be kept cool during exposure.

The second metal exposure container tested is illustrated in Fig. 7. It consisted of a square steel chamber inserted in a metal pipette. Top and bottom of the container were covered with sheet rubber diaphragms. The total volume of the container was 3.5 milliliters. A thermocouple was inserted in the container and as soon as the temperature began to rise cooling was effected

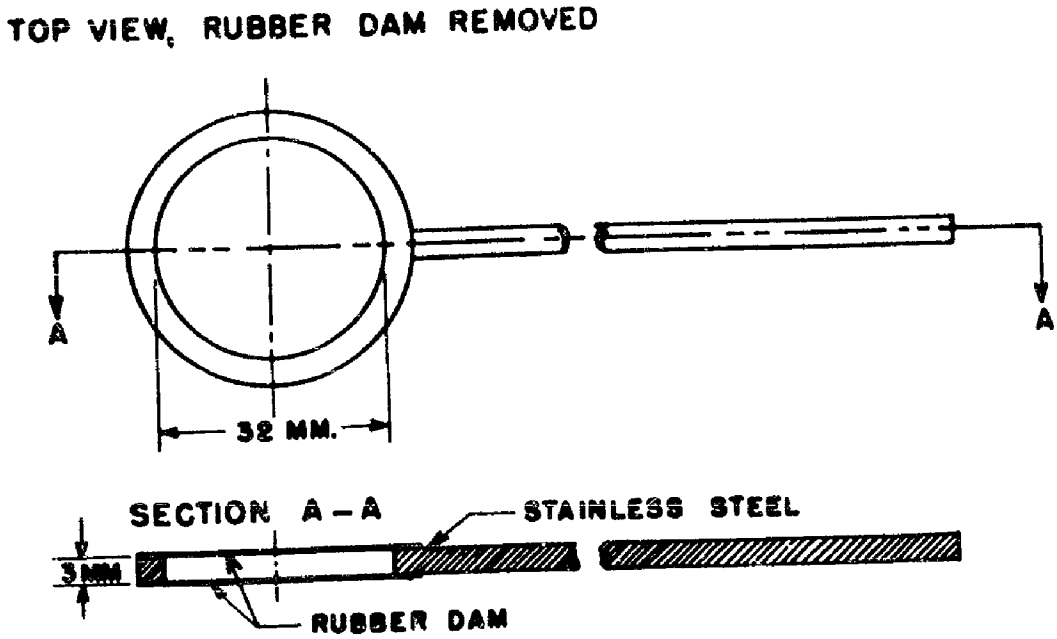


Fig. 6 Exposure container.

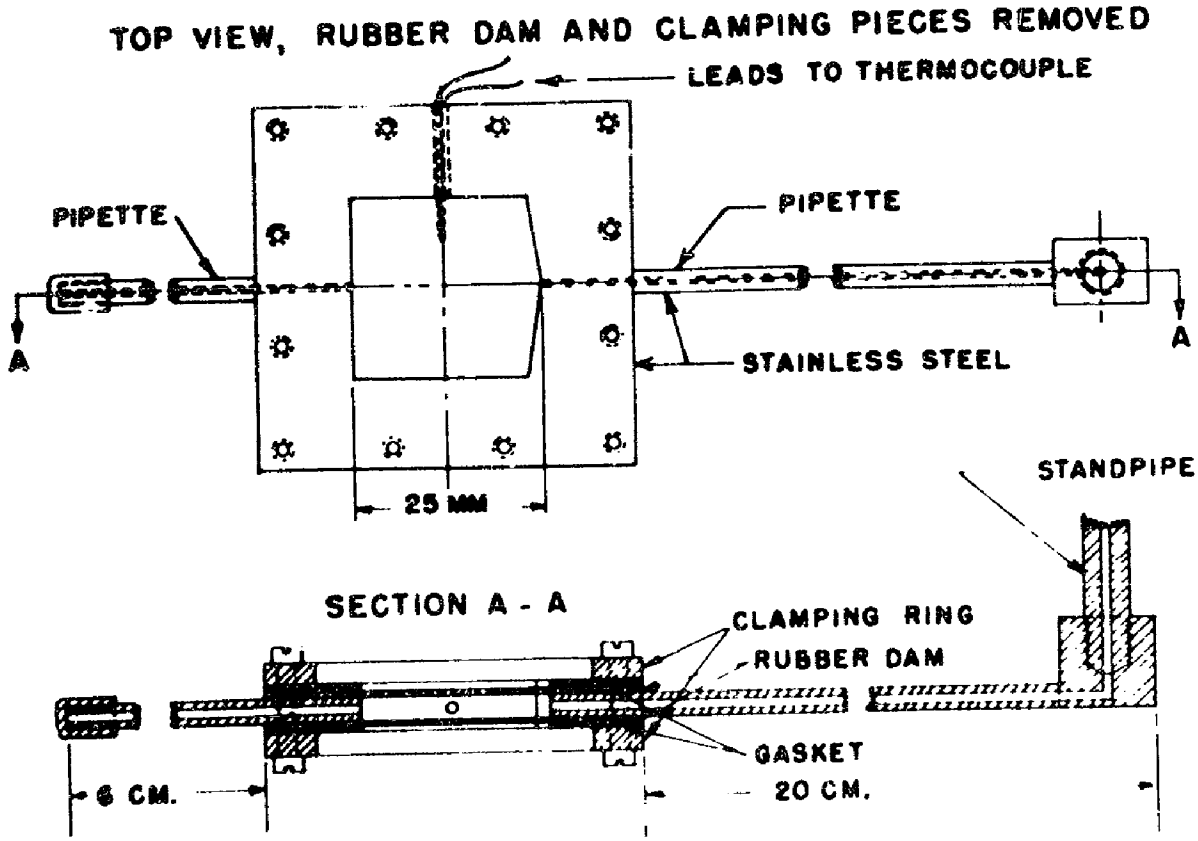


Fig. 7 Exposure container.

by plunging the entire container into an ice bath. There was some indication that the presence of an air bubble during exposure resulted in lethal effects. The container was deemed unsatisfactory and discarded because some of the suspension remained in the pipette and was unexposed, it was difficult to clean thoroughly between exposures, and the use of rubber rendered it unsuitable for sterilization with heat.

The tubular container illustrated in Fig. 8 was next tried. It was constructed of brass and lucite and a sheet rubber diaphragm was employed. This was quickly discarded because the brass was found to be toxic to the bacteria. A container of similar design, illustrated in Fig. 9, but of greater volume was employed concurrently with the one shown in Fig. 8. Both sheet rubber and a thin brass sheet were employed as diaphragms. The metal sheet quickly shattered when exposed to sound and the toxic effects of the brass made it unsuitable.

The stainless steel container illustrated in Fig. 10 was next used. The volume of this container was approximately 4 milliliters. Sheet rubber first was used as a diaphragm for this container, but an extensive search had shown that the plastic Koroseal was better adapted to this purpose than rubber. This plastic not only transmitted the sound waves as well as the rubber but could be autoclaved with little or no ill effects. Consequently Koroseal was used exclusively in all further work. Cooling was effected by attaching a lucite cup filled with ice to the top of the container. The temperature was checked with a thermocouple. Although satisfactory in some respects the volume was not large enough to permit continuous exposure with periodic removal of samples.

In order to test the possibility that a tubular container approximately 5 cm in diameter and 25 cm in length might prove satisfactory, from the physical standpoint, a lucite container of these dimensions was made and studied. As these dimensions proved suitable from the acoustical standpoint the stainless steel tube illustrated in Fig. 11 was built and has been used in much of the work with the siren. The Koroseal diaphragm is held in place by rubber bands placed around the perimeter of the tube. A lucite cup, filled with ice, screws onto the lower part of the tube. This effectively holds the contents of the tube to room temperature or below.

Two modifications, Figs. 12 and 13, of the stainless steel tube have also been used. The modified tubes are both similar in design to the tube just described but are 1.25 cm in diameter and 5 cm in length. The first of these modifications, Fig. 12, required the use of 3.3 milliliters of bacterial suspension overlayed with 5.5 milliliters of sterile mineral oil. This tube also has a lucite coolant jacket. The tube diaphragm forms the

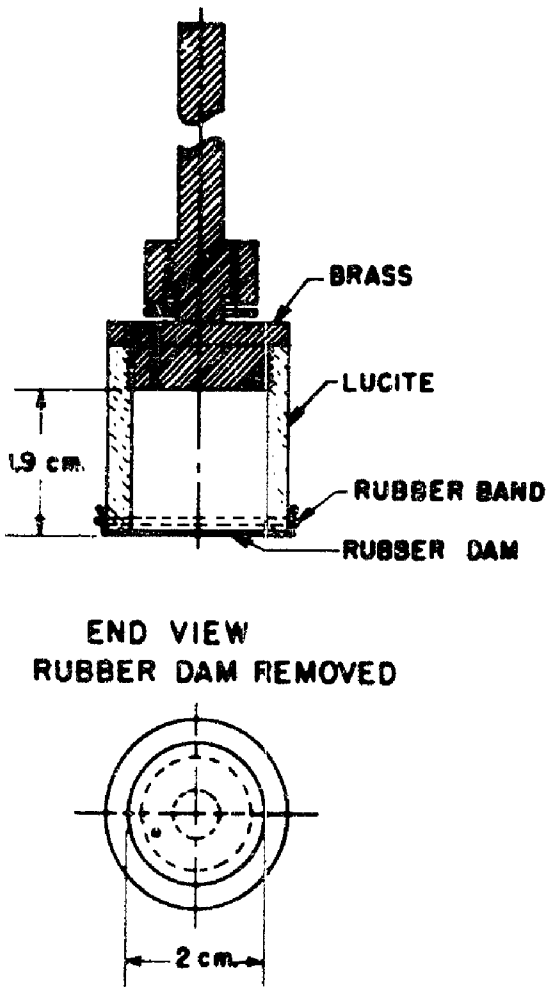


Fig. 8 Exposure container.

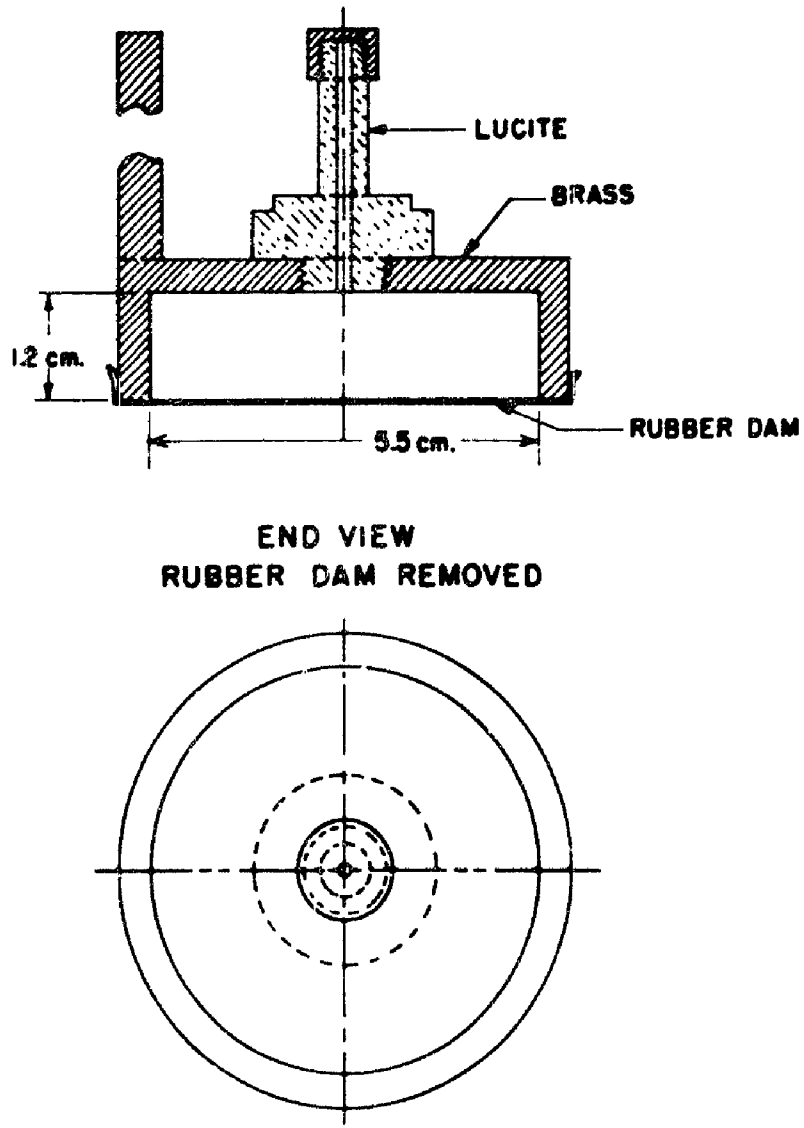


Fig. 9 Exposure container.

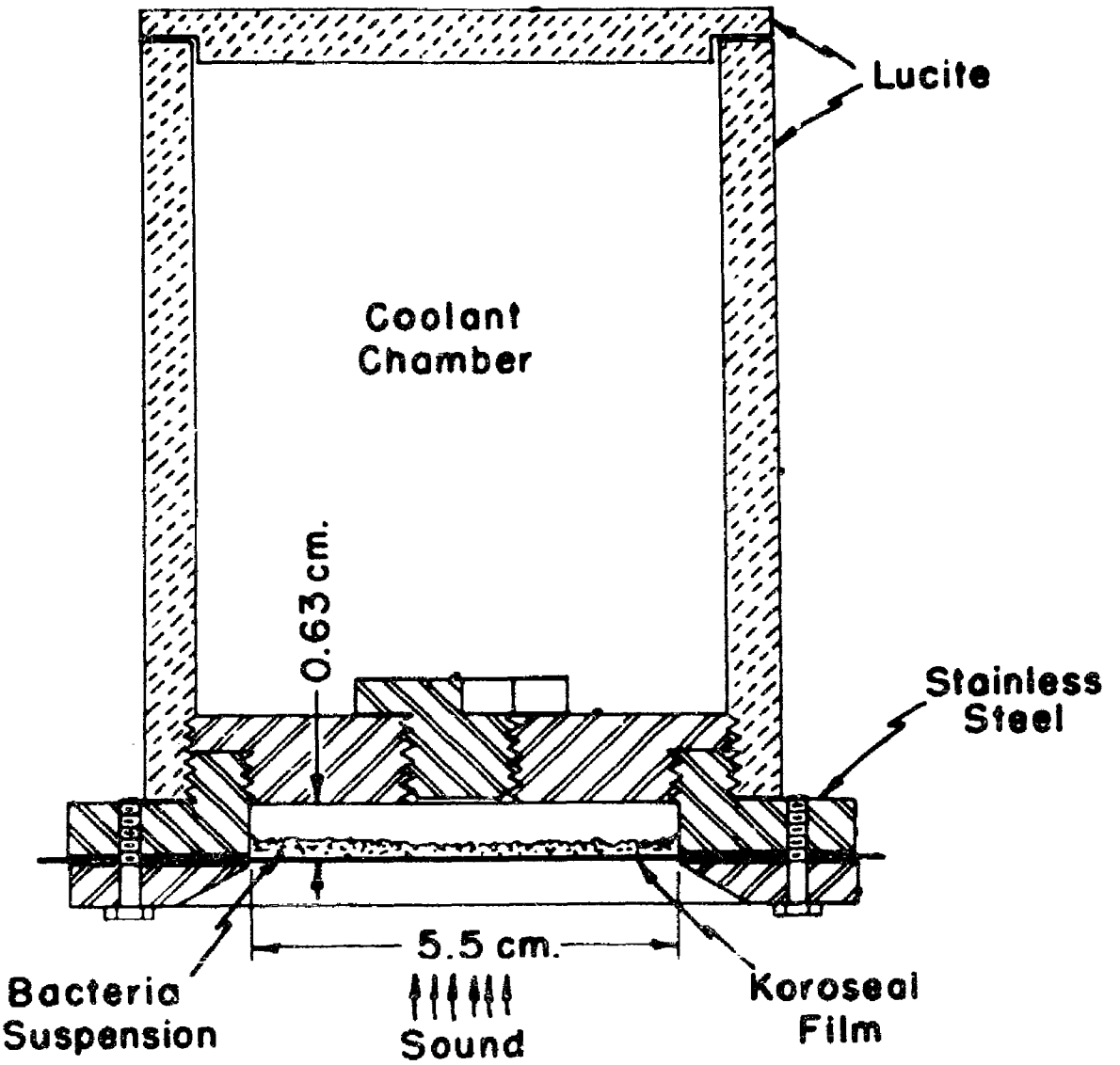


Fig. 10 Exposure container with lucite coolant jacket.

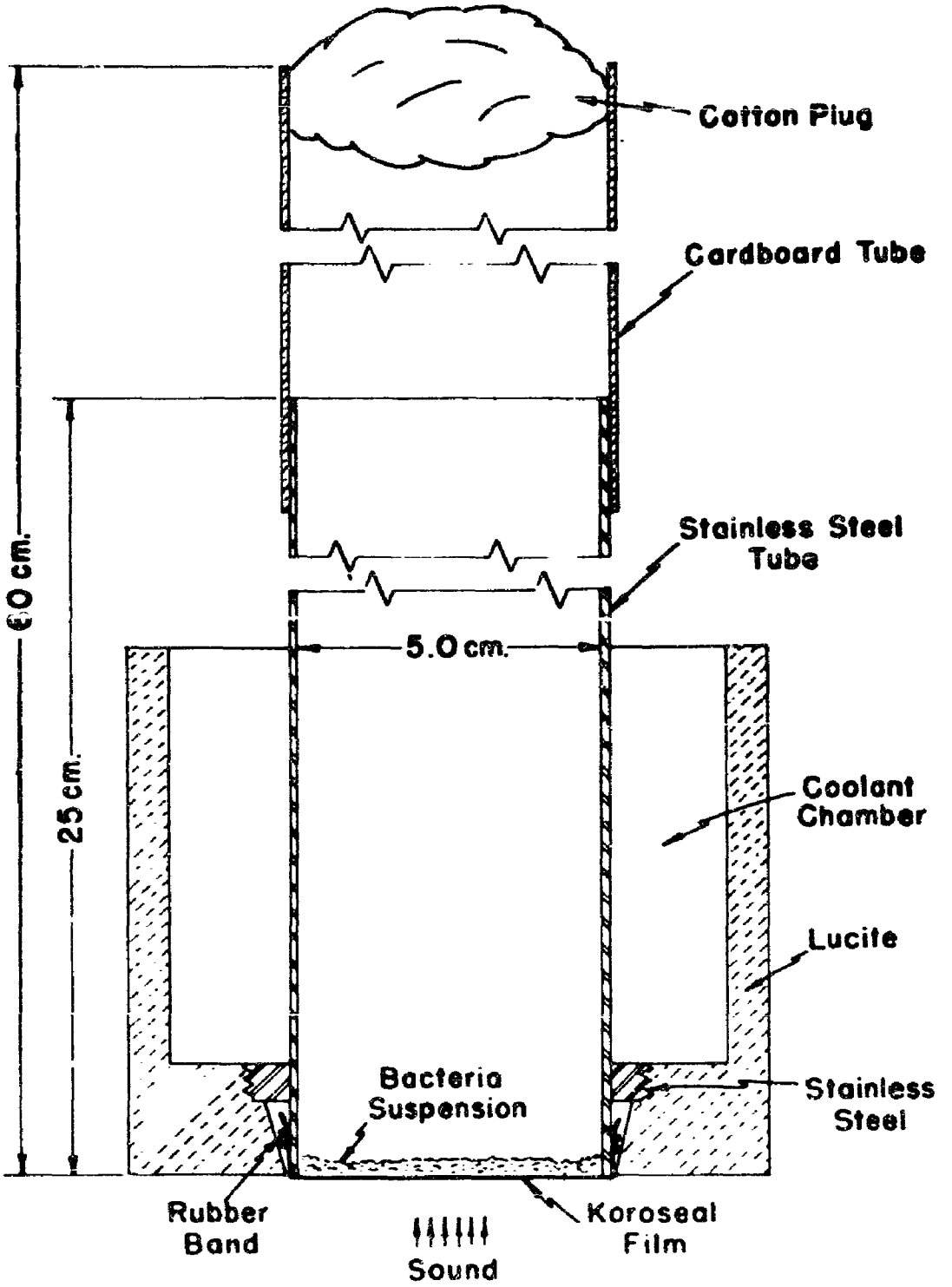


Fig. 11 Exposure container with lucite coolant jacket.

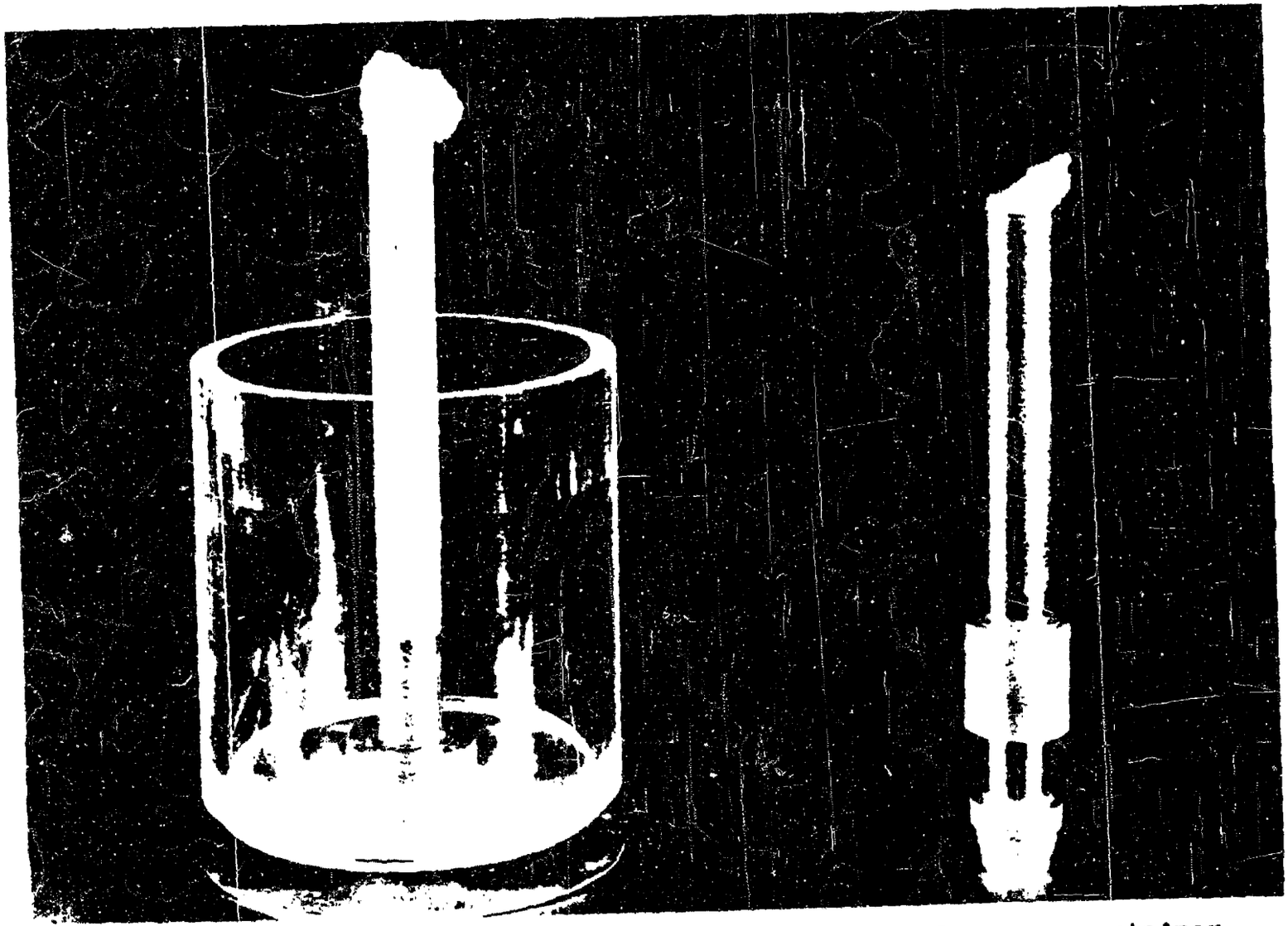


Fig. 12 Exposure container with lucite coolant jacket and resonant cavity.

Fig. 13 Exposure container

upper end of a resonant cavity designed to give a pressure loop at the diaphragm. The diameter of the cavity, 1.25 cm, is so small that the gain in pressure amplitude is only about 3 db (40%). Due to mixing of the bacterial suspension and the oil, especially during the longer exposure periods, this container was deemed unsatisfactory. The container was then cut, Fig. 13, so that a Koroseal diaphragm could be inserted at the top of the 3.3 milliliters of suspension; this eliminated the need for mineral oil. With this container it is possible to place the suspension in the lower part of the tube and thus expose the cells in a sound field of maximum sound pressure and minimum particle velocity or to place the suspension above the second diaphragm and conduct the exposure in the region of minimum sound pressure and maximum particle velocity. The other portion of the tube, in each case, contained water.

These exposure containers have been described in The Technical Report #52-177 "Controlled Sonic Irradiation of Living Organisms" by W. L. Nyborg.

DEVELOPMENT OF EXPOSURE TECHNIQUES

In the course of the development of a suitable exposure container for use with the siren, it was necessary to devise new techniques for the exposure of bacterial cultures as each radical change in the container was made. With the development of the stainless steel tube shown in Fig. 11 a technique was devised suited to this container. Thirty milliliters of the bacterial suspension were pipetted into the exposure container. The pipette was placed at the bottom of the container to avoid the possibility of drops clinging to the sides. Investigation showed that any volume under 7 milliliters would be turbulent. This would greatly complicate the sound field measurements, and possibly also introduce non-acoustic effects. In order to insure sufficient volume to facilitate the removal of numerous samples and still maintain a final volume in excess of 7 milliliters, the 30 milliliter amount was chosen. From the 30 milliliter volume, a control sample of 2 milliliters was removed, before exposure, and placed in a sterile test tube. The exposure container was held by a clamp and placed with the diaphragm 15 cm above the face of the siren as illustrated in Fig. 2. Great care was used to center the container directly over the center of the siren with the diaphragm perfectly parallel with the face of the siren. At various time intervals, the siren was stopped momentarily and a 2 milliliter sample was removed to a sterile test tube to be used in the bacteriological studies. At the conclusion of the exposures, a 2 milliliter sample was taken from the original suspension in the flask and used for a second control. If more than an hour elapsed before bacteriological testing, the test tubes were placed in a refrigerator and held there until testing was started.

In the experiments in which nutrient broth was used as the suspending medium, the samples were immediately chilled by placing the test tubes in cracked ice, to prevent any growth of the microorganisms. The tubes were removed to a refrigerator before the suspension had begun to freeze.

A special technique was necessary for filling the exposure tube illustrated in Fig. 12 and also for removing the sample from this tube. The tube is filled by pipetting 3.3 milliliters of suspension into the bottom of the tube and then carefully layering 5.5 milliliters of sterile mineral oil on top of the suspension. Following exposure the sample is removed by puncturing the Koroseal diaphragm with a sterile hypodermic needle and allowing the suspension to run into a sterile syringe.

The modification of this tube illustrated in Fig. 13 is filled by pipetting 3.3 milliliters of suspension into the lower section of the tube and then carefully stretching the Koroseal diaphragm across the opening so that there are no bubbles visible in the suspension. Again the sample is removed by puncturing the diaphragm with a hypodermic needle. When the suspension is placed in the area of minimum sound pressure, maximum particle velocity, the suspension is simply pipetted into the container with the top diaphragm in place and the sample is removed with a pipette.

RESULTS OF EXPOSURE OF VARIOUS SPECIES OF MICROORGANISMS IN THE SOUND FIELD OF THE SIREN

During the development period of exposure containers and techniques Serratia marcescens was employed as the test organism. Following the design and construction of the 2 in. stainless steel container for use with the siren, a study was made of possible effects of the sound field on this microorganism. The effects of sound on Micrococcus pyogenes var. aureus were investigated concurrently with Ser. marcescens. Preliminary studies indicated significant lethal effects when M. pyogenes var. aureus was irradiated in the sound field of the siren if the latter was operated at a frequency of 6.0 or 6.4 kc and a free field intensity of 168 db was maintained. As any appreciable deviation from this frequency proved less effective in the destruction of this organism, a frequency of 6.0 or 6.4 kc was used with all the organisms studied.

Serratia marcescens. A study of the growth curve of this organism indicated that, grown in nutrient broth at room temperature, the logarithmic growth phase began at approximately 5 hr after inoculation and extended for a period of approximately 25 hr. Decline in numbers of viable cells was found to begin between 36 and 42 hr following inoculation. On the basis of these data, 24 and 48 hr cultures were used in the studies.

Typical plate counts obtained in a series of exposures of pigmented cells of Ser. marcescens exposed at a frequency of 6.4 kc and a free field intensity of approximately 168.5 db* are presented in Table 1. All of the examples recorded checked within an experimental error of 10% on the duplicate controls and within less than 10% on the duplicate plates for each exposure time. One exposure, not included in this table, ran a total time of 120 min with results similar to those reported. All plates were countable at the same dilution; and there was no decrease within an experimental error of 20% and no steady decrease within an experimental error.

Data typical of those obtained under the same exposure conditions but employing a non-pigmented culture of Ser. marcescens are summarized in Table 2. It was thought that the pigment might have some protective effect but the results obtained failed to confirm this, no significant lethal effects were observed in exposures as long as 120 min.

Data obtained from exposure of organisms cultured in nutrient broth and exposed in the broth in which grown are presented in Table 3. It is assumed that in the case of the 24 hr culture the cells were actively growing at the time of irradiation. These data do not indicate any significant destructive effect by the scund waves employed.

Exposed suspensions and progeny of exposed suspensions were examined periodically in an effort to detect morphological or cultural variations, but no variations were found. Likewise no deviation in the percentage of rough variants on the plates was noted.

The data presented indicate that under the conditions of exposure used, the sound waves had no significant effect on the strain of Ser. marcescens employed.

Salmonella typhosa. Conditions of exposure of this microorganism were the same as those used for Ser. marcescens except that the exposure container did not include the coolant chamber shown in Fig. 11. The temperature was checked by a thermocouple and did not exceed 37°C. Due to the pathogenic nature of the organism, the whole exposure was conducted in a plywood chamber. The box was used only for S. typhosa and could be easily disinfected. Exposures of 90 min produced no detectable lethal effects on this organism as shown in Table 4. As in the case of Ser. marcescens, exposed samples were examined periodically for changes of a morphological or cultural nature and none were noted.

* All sound levels in this section ± 0.5 db

Table 1

Plate counts of pigmented cultures of Serratia marcescens in physiological saline exposed in sound field produced by siren, frequency 6.4 kc, free field intensity 168.5 db.

Exposure	Age of culture hr	Exposure time min	Plate count cells/milliliter
A	24	0	29,000,000
		5	38,000,000
		10	40,000,000
		20	38,000,000
		30	33,000,000
		40	36,000,000
		50	29,000,000
		60	34,000,000
		90	31,000,000
B	48	0	43,000,000
		5	39,000,000
		10	41,000,000
		20	35,000,000
		30	43,000,000
		40	41,000,000
		50	31,000,000
		60	26,000,000
		90	43,000,000

Table 2

Plate counts of non-pigmented cultures of Serratia marcescens in physiological saline exposed in sound field produced by the siren, frequency 6.4 kc, free field intensity 168.5 db.

Exposure	Age of culture hr	Exposure time min	Plate count* cells/ milliliter	Plate count* cells/ milliliter
A	24	0	65,000,000	50,000,000
		30	74,000,000	55,000,000
		40	68,000,000	--
		50	74,000,000	--
		60	9,000,000	65,000,000
		90	--	50,000,000
		120	--	44,000,000
B	48	0	28,000,000	24,000,000
		30	26,000,000	26,000,000
		40	--	20,000,000
		50	--	25,000,000
		60	28,000,000	20,000,000
		70	--	19,000,000
		90	31,000,000	--
		120	27,000,000	--

* Separate exposures

Table 3

Plate counts of pigmented cultures of Serratia marcescens exposed in nutrient broth, in which grown, in sound field produced by siren, frequency 6.4 kc, free field intensity 168.5 db.

Exposure	Age of culture hr	Exposure time min	Plate count* cells/ milliliter	Plate count* cells/ milliliter
A	24	0	370,000,000	230,000,000
		20	390,000,000	230,000,000
		40	370,000,000	280,000,000
		60	420,000,000	290,000,000
		80	--	240,000,000
B	48	0	680,000,000	
		20	750,000,000	
		40	680,000,000	
		60	570,000,000	
		80	530,000,000	

* Separate exposures

Table 4

Plate counts of suspension of Salmonella typhosa exposed in sound field produced by the siren, frequency 6.4 kc, free field intensity 168.5 db.

Exposure	Age of culture hr	Exposure time min	Plate count cells/milliliter
A	24	0	28,000,000
		30	21,000,000
		60	28,000,000
		90	26,000,000
B	42	0	23,000,000
		30	31,000,000
		60	16,000,000
C	48	0	39,000,000
		30	35,000,000
		60	41,000,000
		90	38,000,000
D*	22	0	560,000,000
		30	560,000,000
		60	550,000,000
		90	470,000,000
E*	44	0	580,000,000
		30	540,000,000
		90	530,000,000

* Exposed in nutrient broth

Streptococcus zymogenes. These microorganisms were exposed to the siren under the conditions previously described and there were no observed lethal effects, or other changes, on exposures of 90 min in the siren sound field.

Micrococcus varians. Due to the slimy nature of the suspension formed by this organism, it was necessary to filter the original suspension through sterile cotton. Otherwise the procedure was the same as used previously. The cells were found to survive 90 min exposure in the sound field of the siren under the conditions used for the other organisms thus far reported.

Micrococcus pyogenes var. aureus. By contrast with the other microorganisms M. pyogenes var. aureus did show significant lethal effects in the sound field. The initial experiments with this microorganism were done to determine a volume of sample suitable from both the bacteriological and physical standpoint. Historically these were carried out before the experiments just described and these results were used in setting up the experiments with other microorganisms.

It was found that great turbulence occurred in volumes of 2 and 3 milliliters during exposure. Although definite motion could still be observed in a volume of 3.5 milliliters it did not occur in larger volumes. Because of a possible relationship between turbulence and lethal effects a series of exposures was made using a volume of 3.5 milliliters in the exposure chamber. Data obtained are presented in Table 5 and indicate significant destruction under the physical conditions obtained with the siren operating at a frequency of 6.0 kc and a free field intensity of 168 db on exposure of 40 min.

From the bacteriological standpoint, a volume of 3.5 milliliters was impractical as a new sample had to be exposed for each plate count. Volumes up to 30 milliliters were tested and although no turbulence could be observed, lethal effects were obtained comparable to those obtained using the smaller volume. A volume of 30 milliliters proved sufficient to satisfy the requirements of interval sampling during exposure.

As preliminary investigations with this microorganism gave indications of lethal effects when exposed to the sound field of the siren, a more extensive investigation was undertaken. Using Strain I, a frequency of 6.0 kc and maintaining a free field intensity of approximately 168.5 db a study was made of the effect of culture age. A 48 hr culture had been used in the preliminary investigations and typical results obtained with the exposure conditions produced by the siren at that time are shown in Table 6 and graphically in Fig. 14. Comparison of experimental results using 24, 36 and 40 hr cultures are presented in Table 7 and graphically in Fig. 15. The younger cultures showed a steady

Table 5

The effect of sound waves produced by the siren on 48 hr cultures of Micrococcus pyogenes var. aureus, Strain I; volume of exposed sample 3.5 milliliters, frequency 6.0 kc, free field intensity 168.5 db.

Exposure	Exposure time min	Plate count cells/milliliter	Per cent killing
A	0	1,200,000	-
	40	760,000	36.2
B	0	500,000	-
	50	78,000	98.5
	60	48,000	99.1
C	0	9,900,000	-
	70	67,000	99.9

decrease in viable cells during exposure as did the 48 hr cultures, except that in the case of the younger cultures a hump appeared in the death curve. Samples obtained after about 30 min exposure gave higher counts than samples obtained with somewhat shorter exposures. On the other hand, as shown in Fig. 14, a rather smooth line was obtained with the 48 hr culture. Although this hump in the curve obtained with the younger cultures may be due in part to some stimulating effect with short exposure, it is probably due to sonic breaking of clumps of cells which escaped dispersion in the preparatory shaking. If the latter is true, it would indicate that with this strain grown under these conditions homogeneity is more readily obtained on the shaker in the case of a 48 hr culture than in the case of a younger culture.

Using a 48 hr culture of Strain I, a frequency of 6.0 kc and a free field intensity of 168 db a comparative study of plate counts and microscopic appearance of exposed cells was made. The plate counts obtained are shown in Table 8 and the microscopic studies in Figs. 16 to 24. It will be noted that as the plate count declines, debris may be seen in the photomicrographs and as the plate count reaches a low level, the number of intact cells is relatively few as compared with intact cells appearing after only short exposures.

A comparison of morphological and physiological characteristics was made of the microorganisms prior to and following exposure. Unexposed cultures, exposed suspensions and progeny of cells surviving exposure were tested for possible changes due to sonic exposure but no changes were noted in the appearance of the intact cells, the physiological characteristics or the rate of physiological activity of the surviving cells.

Strain II, a pathogenic strain of M. pyogenes var. aureus employed in this investigation, was tested for changes in hemolytic activity and coagulase production due to sonic exposure. Bovine blood agar plates were streaked with unexposed cultures, suspensions exposed for varying time intervals, cultures obtained from cells surviving exposure, cell-free filtrates of exposed suspensions and cell-free filtrates of unexposed suspensions. Exposed suspensions and progeny of cells surviving exposure retained their ability to produce beta hemolysis on blood agar plates. Cell-free filtrates of unexposed suspensions produced no hemolysis but the cell-free filtrates of suspensions exposed to the sound field for only a few minutes were found to produce beta hemolysis. This seems to indicate, under the physical conditions maintained during these exposures, that hemolysins were released from the destroyed cells.

The same substances tested for hemolytic activity were tested for coagulase production. Exposed suspensions and progeny of surviving cells remained coagulase positive. Cell-free filtrates

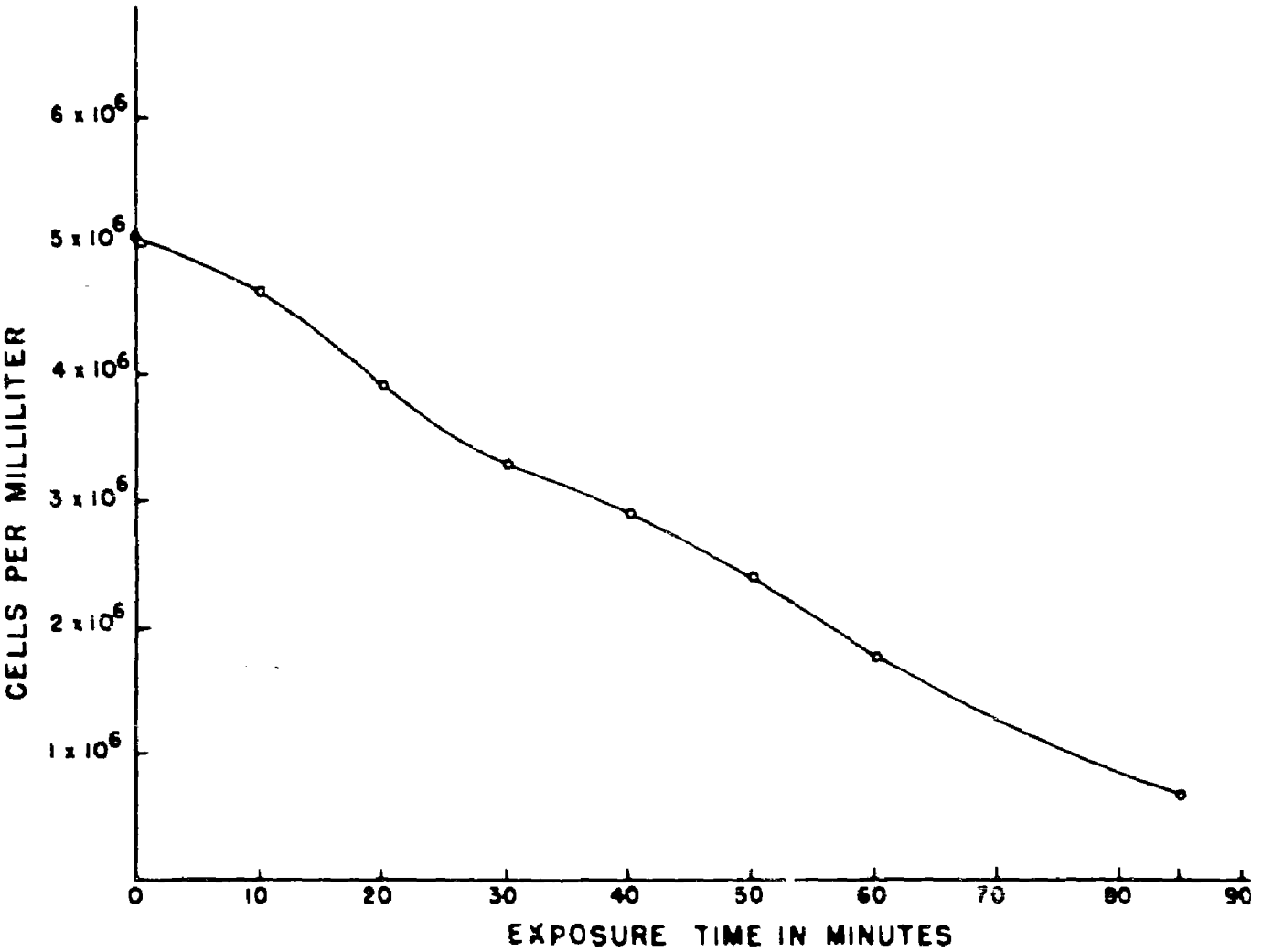


Fig. 14 Plate counts made at intervals during the exposure of a 48 hr culture of Micrococcus pyogenes var. aureus, Strain I, frequency 6.4 kc, free field intensity 168.5 db.

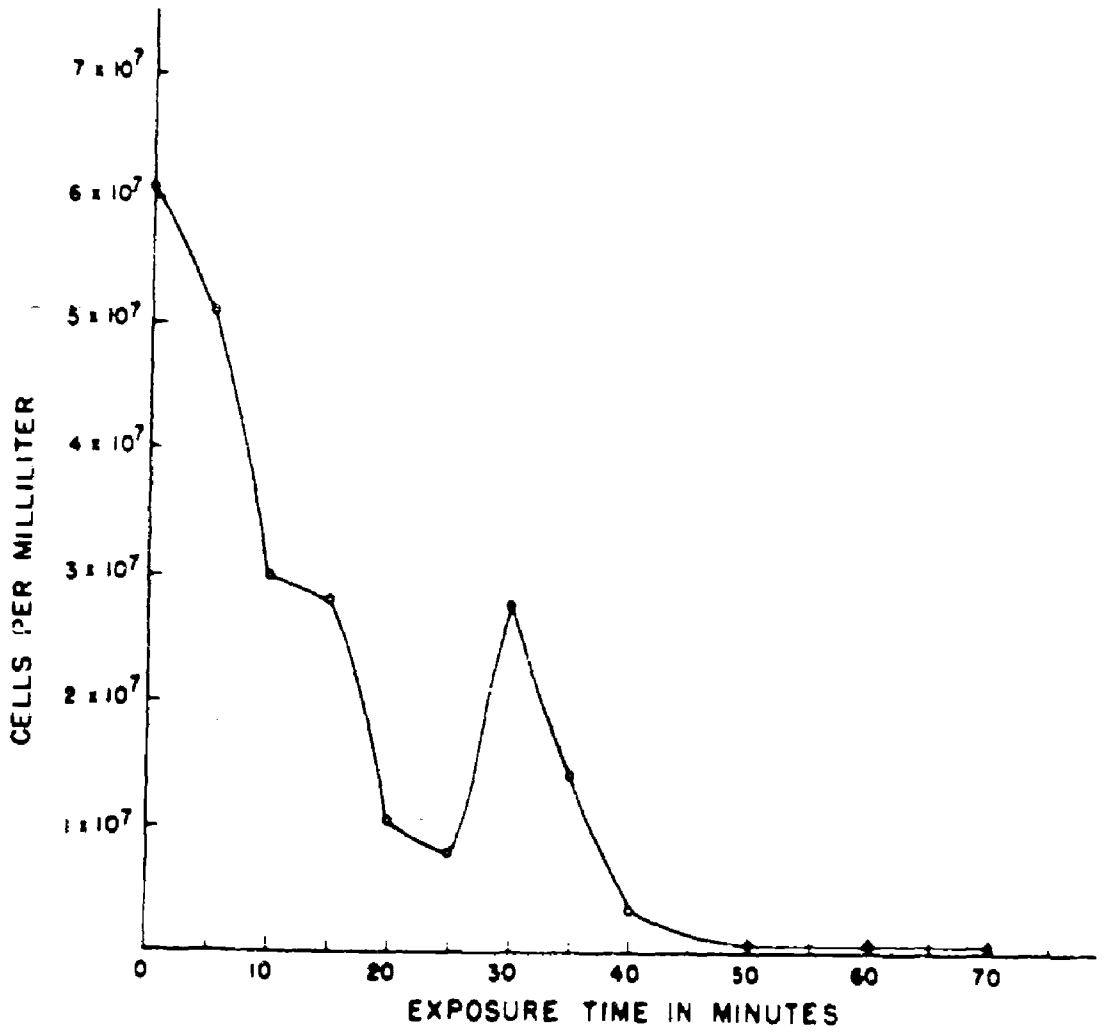


Fig. 15 Plate counts made at intervals during the exposure of a 36 hr culture of Micrococcus pyogenes var. aureus, Strain I, frequency 6.4 kc, free field intensity 168.5 db.

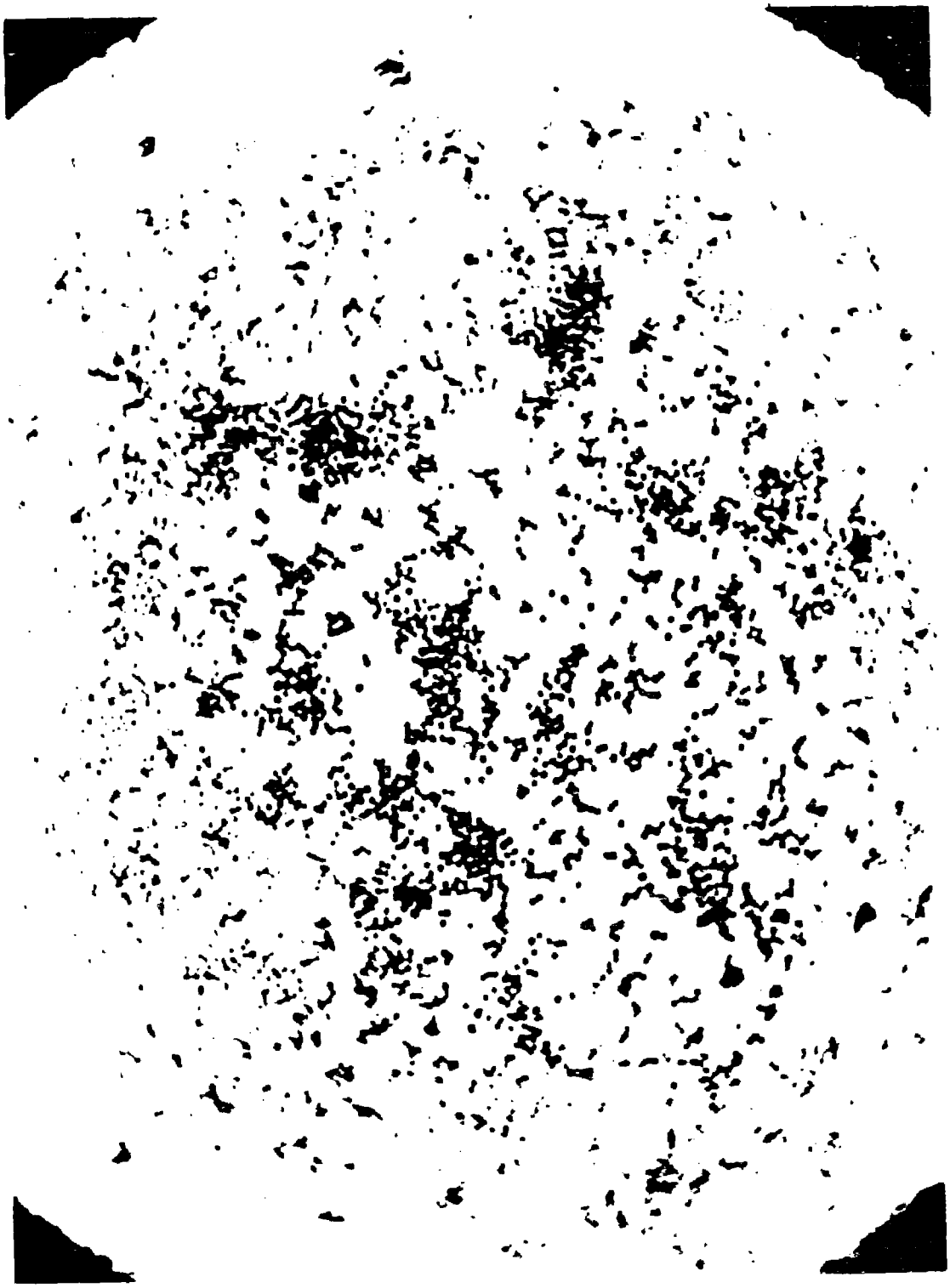


Fig. 16 Photomicrograph of an unexposed 48 hr culture of Micrococcus pyogenes var. aureus, Strain I.

Fig. 17 Photomicrograph of a 48 hr culture of Micrococcus pyogenes var. aureus, Strain I; exposed to the sound field of the siren, frequency 6.4 kc, free field intensity 168.5 db for 10 min.

Fig. 18 Photomicrograph of a 48 hr culture of Micrococcus pyogenes var. aureus, Strain I; exposed to the sound field of the siren, frequency 6.4 kc, free field intensity 168.5 db for 20 min.

Fig. 19 Photomicrograph of a 48 hr culture of Micrococcus pyogenes var. aureus, Strain I; exposed to the sound field of the siren, frequency 6.4 kc, free field intensity 168.5 db for 30 min.

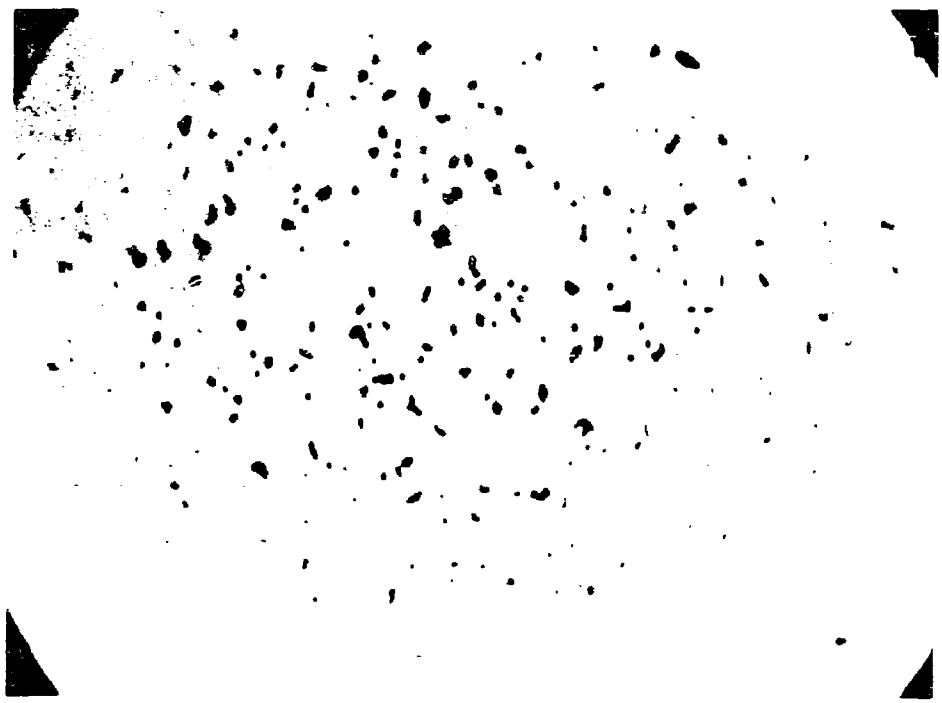


Fig. 20 Photomicrograph of a 48 hr culture of Micrococcus pyogenes var. aureus, Strain I; exposed to the sound field of the siren, frequency 6.4 kc, free field intensity 168.5 db for 40 min.

Fig. 21 Photomicrograph of a 48 hr culture of Micrococcus pyogenes var. aureus, Strain I; exposed to the sound field of the siren, frequency 6.4 kc, free field intensity 168.5 db for 50 min.

Fig. 22 Photomicrograph of a 48 hr culture of Micrococcus pyogenes var. aureus, Strain I; exposed to the sound field of the siren, frequency 6.4 kc, free field intensity 168.5 db for 60 min.

Fig. 23 Photomicrograph of a 48 hr culture of Micrococcus pyogenes var. aureus, Strain I; exposed to the sound field of the siren, frequency 6.4 kc, free field intensity 168.5 db for 70 min.

Fig. 24 Photomicrograph of a 48 hr culture of Micrococcus pyogenes var. aureus, Strain I; exposed to the sound field of the siren, frequency 6.4 kc, free field intensity 168.5 db for 80 min.

Table 6

The effect of sound waves produced by the siren on 24, 36 and 40 hr cultures of Micrococcus pyogenes var. aureus, Strain I; volume of exposed sample 30 milliliters, frequency 6.0 kc, free field intensity 168.5 db.

Exposure	Age of culture hr	Exposure time min	Plate count cells/milliliter	Per cent killing
A	24	0	21,000,000	-
		5	18,000,000	13.9
		10	12,000,000	42.9
		15	11,000,000	49.1
		20	4,400,000	79.1
		25	4,100,000	80.5
		30	12,000,000	44.8
		35	5,000,000	76.2
		40	1,900,000	91.0
B	36	0	61,000,000	-
		5	51,000,000	16.5
		10	30,000,000	50.9
		15	28,000,000	54.0
		20	10,000,000	83.2
		25	8,000,000	86.9
		30	28,000,000	54.6
		35	14,000,000	77.4
		40	3,200,000	94.8
		50	850,000	98.6
		60	840,000	98.7
		70	600,000	99.1
C	40	0	9,000,000	-
		5	8,100,000	10.0
		10	4,700,000	48.4
		15	4,400,000	51.2
		20	2,000,000	77.8
		30	5,300,000	41.2

Table 7

The effect of sound waves produced by the siren on 48 hr cultures of Micrococcus pyogenes var. aureus, Strain I; volume of exposed sample 30 milliliters, frequency 6.0 kc, free field intensity 168.5 db.

Exposure	Exposure time min	Plate count cells/milliliter	Per cent killing
A	0	3,000,000	-
	1	3,000,000	0
	2	3,000,000	0
	3	2,900,000	3.4
	4	2,900,000	3.4
	5	2,800,000	6.7
B	0	3,000,000	-
	10	1,900,000	36.7
	20	1,600,000	46.7
	30	1,500,000	50.0
	40	1,200,000	60.0
	50	240,000	92.0
	60	160,000	94.7
C	0	5,000,000	-
	10	4,700,000	6.0
	20	3,900,000	22.0
	30	3,300,000	34.0
	40	2,900,000	42.0
	50	2,400,000	52.0
	60	1,800,000	64.0
	85	240,000	95.2

Table 8

The effect or sound waves produced by the siren on a 48 hr culture of Micrococcus pyogenes var. aureus, Strain I; plate counts made in the course of a microscopic study shown in Figs. 17 to 25, frequency 6.0 kc, free field intensity 168.5 db.

Exposure time min	Plate count cells/milliliter	Per cent killing
0	13,000,000	-
10	12,000,000	9.8
20	10,000,000	20.4
30	9,400,000	26.6
40	8,000,000	37.5
50	7,100,000	44.6
60	3,200,000	75.0
70	190,000	85.4
80	100,000	92.2
90	91,000	99.3
100	8,400	99.9

of suspensions exposed for only 10 min were found to contain enough coagulase to be detected in 0.5 milliliter of the filtrate. The cell-free filtrates of unexposed suspensions were coagulase negative.

Destruction of the organism having been indicated by plate counts and by microscopic examination, a serological study of the filtrates from exposed suspensions of M. pyogenes var. aureus, Strain II, was undertaken in an effort to obtain further confirmation of physical disruption. A frequency of 6.4 kc was used for this and subsequent work instead of the frequency of 6.0 kc formerly employed as physical measurements had shown that the siren had less tendency to vary at 6.4 kc than at 6.0 kc. A free field intensity of 168.5 db was maintained, however, so the change in physical conditions was not great.

The filtrates used for the precipitin test were obtained from suspensions exposed to a frequency of 6.4 kc and a free field intensity of 168.5 db for 90 min. These filtrates as well as filtrates from unexposed suspensions were tested with both homologous antiserum and normal serum. An acid extracted antigen prepared from this strain was also tested against the homologous antiserum. The results obtained are shown in Table 9.

Normal serum did not react with filtrates of either exposed or unexposed suspensions. On the other hand a precipitin titer of 1:1024 was obtained with the use of filtrates from exposed suspensions and the homologous antiserum, in contrast with no reaction in the series of tubes containing unexposed filtrates and the homologous antiserum. The exposed filtrate yielded a higher titer than did the acid extracted antigen employed as a control. From these data it is concluded that, under the conditions obtained at that time, exposure of this organism resulted in the release of significant concentrations of antigenic fragments of cells in the suspending medium. This is submitted as further evidence of cell destruction.

Following this a study was made to determine the effects of variation of exposure time, free field intensity, and the frequency. In the earlier portion of this research exposure time was investigated by means of interval sampling and plating, these earlier studies had not included serological examinations of the filtrates. A series of studies was conducted, therefore, in which plate counts and serological tests were made on samples obtained at intervals of 30 min during 90 min exposures at a frequency of 6.4 kc and a free field intensity of 168.5 db. Typical of all the data obtained in this series are the results of one exposure presented in Table 10. The data show that under the conditions obtained less than 15% of the organisms are destroyed in the first 30 min of exposure, that about two thirds are destroyed in the first 60 min and that destruction of at least 99% may be expected in a period of 90 min.

Table 9

Determination of the precipitin titers of filtrates of suspensions of Micrococcus pyogenes var. aureus, Strain II; exposed to sound waves produced by the siren for 90 min and of filtrates of unexposed suspensions of the organism, frequency 6.4 kc, free field intensity 168.5 db.

Antigen dilutions	Exposed filtrate with undiluted antiserum	Exposed filtrate with undiluted normal serum	Unexposed filtrate with undiluted antiserum	Unexposed filtrate with undiluted normal serum	Acid extracted antigen with undiluted antiserum
1:1	+	-	-	-	+
1:2	+	-	-	-	+
1:4	+	-	-	-	+
1:8	+	-	-	-	+
1:16	+	-	-	-	+
1:32	+	-	-	-	+
1:64	+	-	-	-	+
1:128	+	-	-	-	+
1:256	+	-	-	-	-
1:512	+	-	-	-	-
1:1024	+	-	-	-	-
1:2048	-	-	-	-	-
1:4096	-	-	-	-	-
1:8192	-	-	-	-	-
1:16384	-	-	-	-	-

Table 10

Relative effect of 30, 60 and 90 min exposures of Micrococcus pyogenes var. aureus, Strain II, to sound waves produced by the siren, frequency 6.4 kc, free field intensity 168.5 db.

Exposure time min	Precipitin titer	Plate count cells/milliliter	Per cent killing
0	0	2,000,000,000	-
30	0	1,700,000,000	13.9
60	1:4	660,000,000	67.0
90	1:1024	200	99.9

Table 11

Relative effect of free field intensities of 165.5, 167.0 and 168.5 db in the exposure of Micrococcus pyogenes var. aureus, Strain II, to sound waves produced by the siren, frequency 6.4 kc, exposure time 90 min.

Free field intensity in db	Precipitin titer	Per cent killing
165.5	0	6.5
167.0	1:2	28.9
168.5	1:1024	99.9

Table 12

Relative effect of frequencies of 5.0, 6.4, 10.0, 15.0 and 24.5 kc in the exposure of Micrococcus pyogenes var. aureus, Strain II, to sound waves produced by the siren, free field intensity 168.5 db, exposure time 90 min.

Frequency in kc	Precipitin titer	Per cent killing
5.0	1:1	16.4
6.4	1:1024	99.9
10.0	1:512	99.9
15.0	1:2	23.0
24.5	0	4.0

The serological results obtained in this series of investigations are of considerable interest. In brief, the data indicate that if killing, as shown by the plate count, is less than 15% under the conditions of this experimental work, no titer is to be expected in the filtrate of exposed suspensions; plate counts indicating killing between 15 and 20% may be expected to be accompanied by titers of 1:1; killings between 20 and 30% by titers of 1:2; killings between 30 and 70% by titers of 1:2 or 1:4 and killings of more than 99% by titers of the order of 1:512 and 1:1024. It is to be understood, of course, that this correlation is dependent upon the potency of the antiserum and the original cell concentration. This seems to indicate that considerable fragmentation, probably after the cells are killed, must occur before sufficient antigenic material is released to give a significant titer.

A study of the effect of variation in free field intensity was made using intensities of 165.5 and 167.0 db as compared with results obtained in a free field intensity of 168.5 db. A frequency of 6.4 kc and 90 min exposure time was used in this study. With the type of container in use at the time of these experiments and the physical limitations of the siren with this method of exposure, a free field intensity of 168.5 db was the maximum that could be maintained. Data obtained at these free field intensities are presented in Table 11. The data indicate that lowering the free field intensity from 168.5 to 167.0 db seriously interferes with destruction of the cells. Further lowering the intensity to 165.5 db results in negligible destruction in an exposure period of 90 min. Precipitin titers confirm the data obtained by plate counts. The conclusion is reached that under the conditions of this experimental study and employing an exposure of 90 min lethal intensities have barely been reached at the 168.5 db level.

On the basis of the above results a free field intensity of 168.5 db and an exposure period of 90 min were chosen for the study of the effect of frequency variation. A series of exposures was made using frequencies of 5.0, 6.4, 10.0, 15.0 and 24.5 kc. Data typical of those obtained in replicate exposures are presented in Table 12. Greatest destruction was obtained at a frequency of 6.4 kc with that obtained at 10.0 kc closely approaching this maximum. Destruction at 5.0 and 15.0 kc was comparatively minor while that obtained at 24.5 kc proved negligible. As in previous studies the plate counts were confirmed by the precipitin titers obtained.

The pronounced effect of frequency found was unexpected and additional physical data were needed to explain the results obtained. It was found that sound pressure distributions within the suspension were by no means the same at all frequencies even

though a constant free field intensity of 168.5 db was maintained. Fig. 25 shows the region within the suspension where the pressure amplitude exceeds 0.08 at. In the case of 5.0, 15.0 and 24.5 kc, this region is relatively small while at a frequency of 6.4 kc this region is greater. With a frequency of 10.0 kc this volume is somewhat less than at 6.4 kc. Therefore, the apparent effects due to frequency variation may well be due to changes in pressure distribution within the suspension.

Following these investigations difficulties developed that have not been explained satisfactorily. Consistent results could no longer be obtained with M. pyogenes var. aureus although the same techniques, with one exception, were followed. Exposures were performed with the diaphragm of the exposure container placed 9, 13, 14 and 15 cm above the face of the siren while in all previous exposures the distance was 15 cm.

Typical results obtained with M. pyogenes var. aureus, Strain I, during this period are shown in Table 13. While in most cases the percentage of cells failing to grow after 30 min exposure is greater than was found in the earlier work with this culture, in three cases the growth obtained following exposure equals that of the unexposed cells. The percentage of cells failing to grow after 90 min exposure approaches the earlier results only once. Also the results obtained from one exposure to another show very little correlation.

These altered results might have been due to three possible causes none of which we can either confirm or eliminate: first, the harmonic content of the siren output might have changed; second, the pressures may not have been the important physical quantity to measure; or third, some sort of a mutation or variation, affecting the ability of the microorganisms to survive in the sound field may have arisen.

M. pyogenes var. aureus, Strain I, was also exposed to the sound field of the siren in a container similar to the one previously used but one-half in. in diameter. In this case 3.3 milliliters of bacterial suspension were placed in the container and 5.5 milliliters of sterile mineral oil were layered over the suspension. Because of the small volume of bacterial suspension interval sampling was not possible and each exposure had to be made on a separate aliquot of the suspension.

The results obtained using this exposure technique are shown in Table 14. Again, in most cases, after exposure to the sound for 30 min about 30% of the cells failed to grow. As there was some mixing of the oil and the suspension, especially during the longer exposure periods, this may account in part for the irregular results obtained.

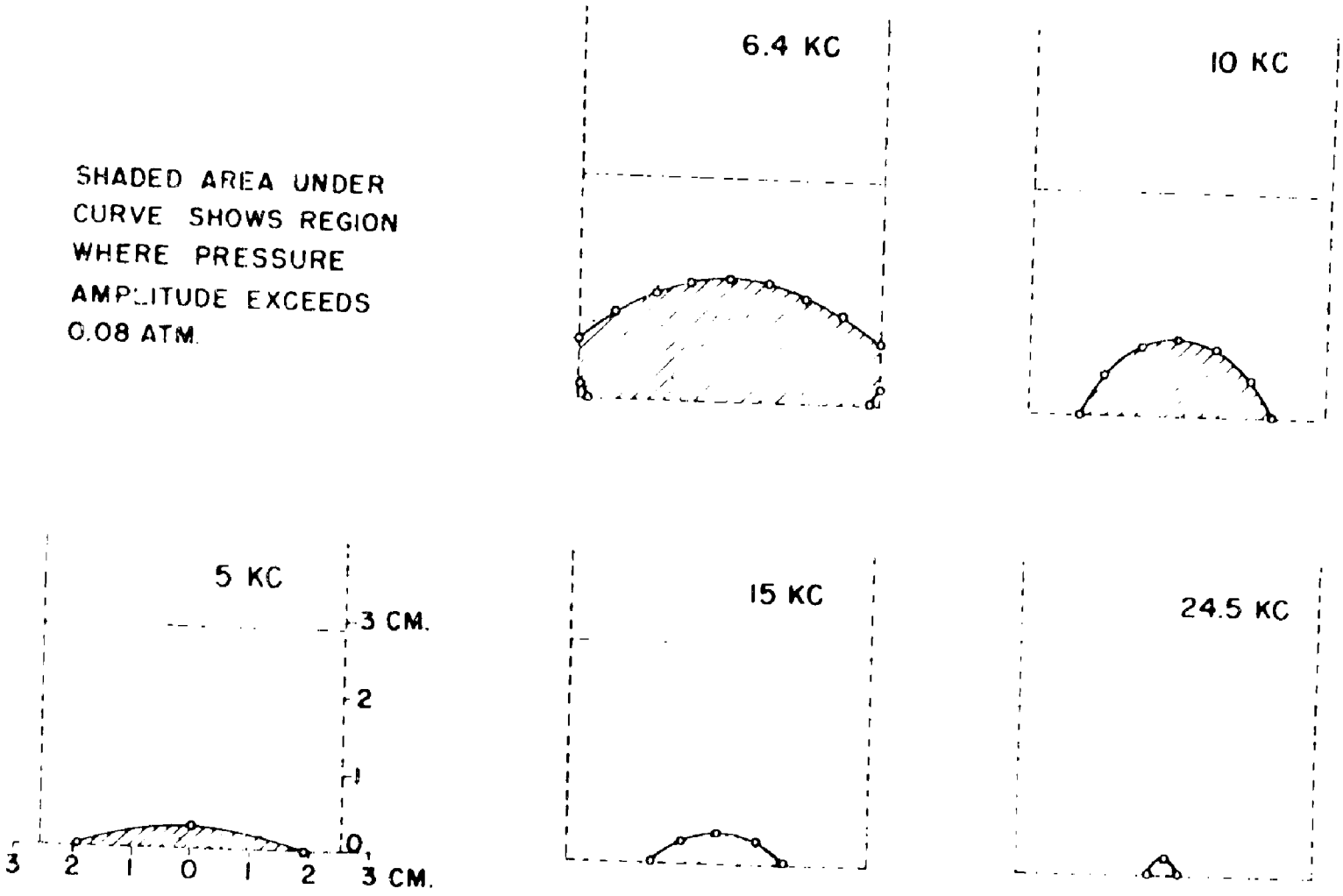


Fig. 25 Pressure amplitude in the exposure container shown in Fig. 11 at frequencies of 5.0, 6.4, 10.0, 15.0 and 24.5 kc, free field intensity 168.5 db.

Table 13

Percentage of cells of Micrococcus pyogenes var. aureus, Strain I, failing to grow following exposure to 6.4 kc sound field produced by the siren.

Distance from siren	Free field intensity	Exposure time in min 30	60	90	120	180
9 cm	168.6 db	--	54	76	--	--
		50	56	65	--	--
		--	--	--	35	--
		--	16	33	50	--
13 cm	168.6 db	14	16	17	38	--
		35	30	29	--	--
		--	--	15	--	--
	170.0 db	34	45	50	--	--
		26	26	26	--	--
		78	--	99	--	--
		40	40	--	--	--
		29	22	53	--	--
		30	43	40	--	--
14 cm	170.0 db	--	0	0	--	--
		0	16	--	--	--
15 cm	168.6 db	--	0	0	--	--
		--	0	--	--	--
		--	0	--	--	62

Table 14

Percentage of cells of Micrococcus pyogenes var. aureus, Strain I, failing to grew following exposure to the siren sound field in the 1.25 cm container using 3.3 milliliters of suspension and 5.5 milliliters of sterile mineral oil, frequency 6.4 kc, free field intensity 182 db, 14 cm from the face of the siren.

Time in min				
15	30	60	90	150
-	32	-	-	-
-	32	-	-	-
-	-	14	-	-
-	44	47	-	-
-	-	21	-	-
-	34	-	34	-
-	37	-	35	-
-	-	-	32	-
-	31	52	74	-
-	-	-	-	25
-	33	-	-	-
65	-	-	-	-
43	24	-	-	-
-	-	85	-	-
9	17	-	-	-
-	-	80	82	-
-	28	-	30	-

This exposure container was then modified, as described in the section on development of exposure containers, so as to eliminate the need for oil. Two microorganisms were exposed in this container. The results obtained with M. pyogenes var. aureus are shown in Table 15 and those with the yeast, Saccharomyces cerevisiae, in Table 16. In the case of M. pyogenes var. aureus about 30% of the cells failed to grow after 30 min exposure in the region of maximum sound pressure minimum particle velocity but again no definite conclusions can be drawn for in one case 90% of the cells failed to grow and in another there was no difference between the number of colonies developing from the exposed and unexposed suspensions. The results after 90 min indicate no greater effect than at 30 min while exposures of 60 min indicate no significant effect resulted under the conditions maintained during these two exposures. In the sound field of minimum pressure maximum particle velocity the results show so much variation that no conclusion can be drawn, except that there seems to have been less effect than that obtained in the maximum sound pressure region.

Using S. cerevisiae as the test microorganism no lethal effect was noted from exposure in the minimum sound pressure maximum particle velocity region. In the field of maximum sound pressure significant effects during 30 min exposure were obtained in only two of the nine exposures and twice effects were obtained in 60 min but in each case the effect was slightly less than in 30 min.

SECTION IV

WHISTLE EXPERIMENTS

THE T TYPE WHISTLE

Whistles of the jet edge resonator (jer) types are discussed in detail in the final report of the PSC Signal Corps Acoustics Project (8). One of the jer whistles was modified to have two resonant cavities, one above and the other below the edge. This T whistle with its attached exposure chamber is illustrated in Figs. 26 and 27. The historical details leading to this form are discussed by Nyborg (7).

In its final form the lower resonant chamber is closed by an adjustable lucite plug, the top by an exposure chamber for liquid samples. The exposure chamber has stainless steel sidewalls and is closed at the bottom by a Koroseal diaphragm which forms the top of the upper resonant air cavity. The diaphragm is held in

Table 15

Percentage of cells of Micrococcus pyogenes var. aureus, Strain I, failing to grow following exposure to the sound field of the siren in the region of maximum sound pressure minimum particle velocity and in the region of minimum sound pressure maximum particle velocity, frequency 6.4 kc, free field intensity 182 db, 14 cm above the face of the siren.

Max sound pressure min particle velocity			Min pressure max particle velocity		
30 min	60 min	90 min	30 min	60 min	90 min
34	-	-	-	-	-
-	-	0	-	-	-
-	10	-	-	-	-
-	10	-	5	-	-
91	-	-	58	-	-
33	-	-	0	-	-
-	-	32	32	-	-
34	-	-	-	15	-
32	-	-	-	11	-
48	-	-	22	-	-
35	-	-	17	-	-
0	-	-	15	-	-
-	-	30	-	-	14
*26	-	-	-	-	-
*27	-	-	-	-	-

* Without resonant cavity

Table 16

Percentage of cells of Saccharomyces cerevisiae failing to grow following exposure to the sound field of the siren in the region of maximum sound pressure minimum particle velocity and in the region of minimum sound pressure maximum particle velocity, frequency 6.4 kc, free field intensity 182 db, 14 cm above the face of the siren.

Max sound pressure min particle velocity			Min pressure max particle velocity		
30 min	60 min	90 min	30 min	60 min	90 min
0	-	-	0	-	-
48	-	-	0	-	-
50	-	-	0	-	-
0	-	-	0	-	-
0	-	-	0	-	-
-	-	-	-	-	0
-	31	-			
-	40	-			
*0	-	-			
*0	-	-			
*-	0	-			
*0	-	-			
*0	-	-			

* Without resonant cavity

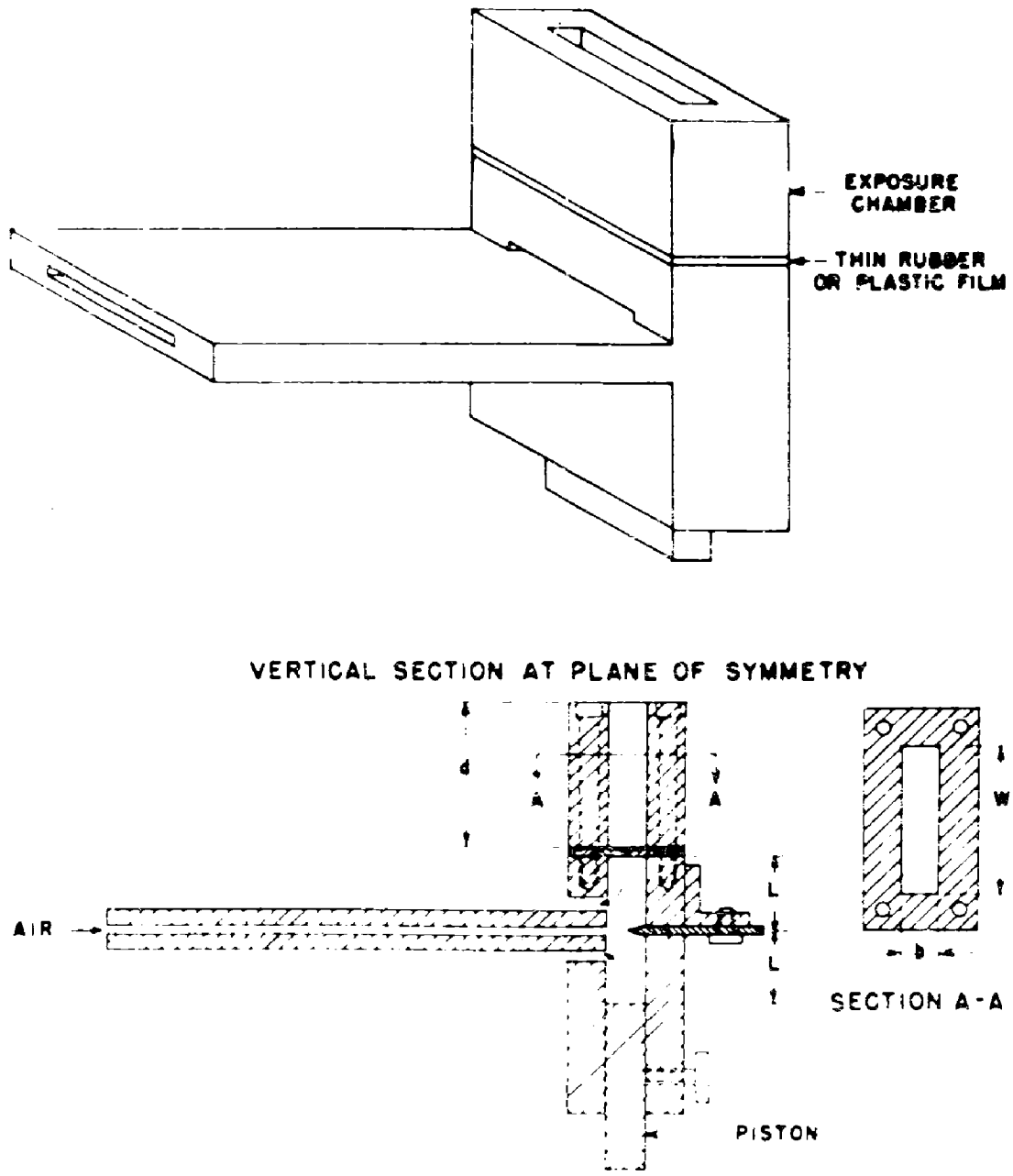


Fig. 26 Type T whistle.

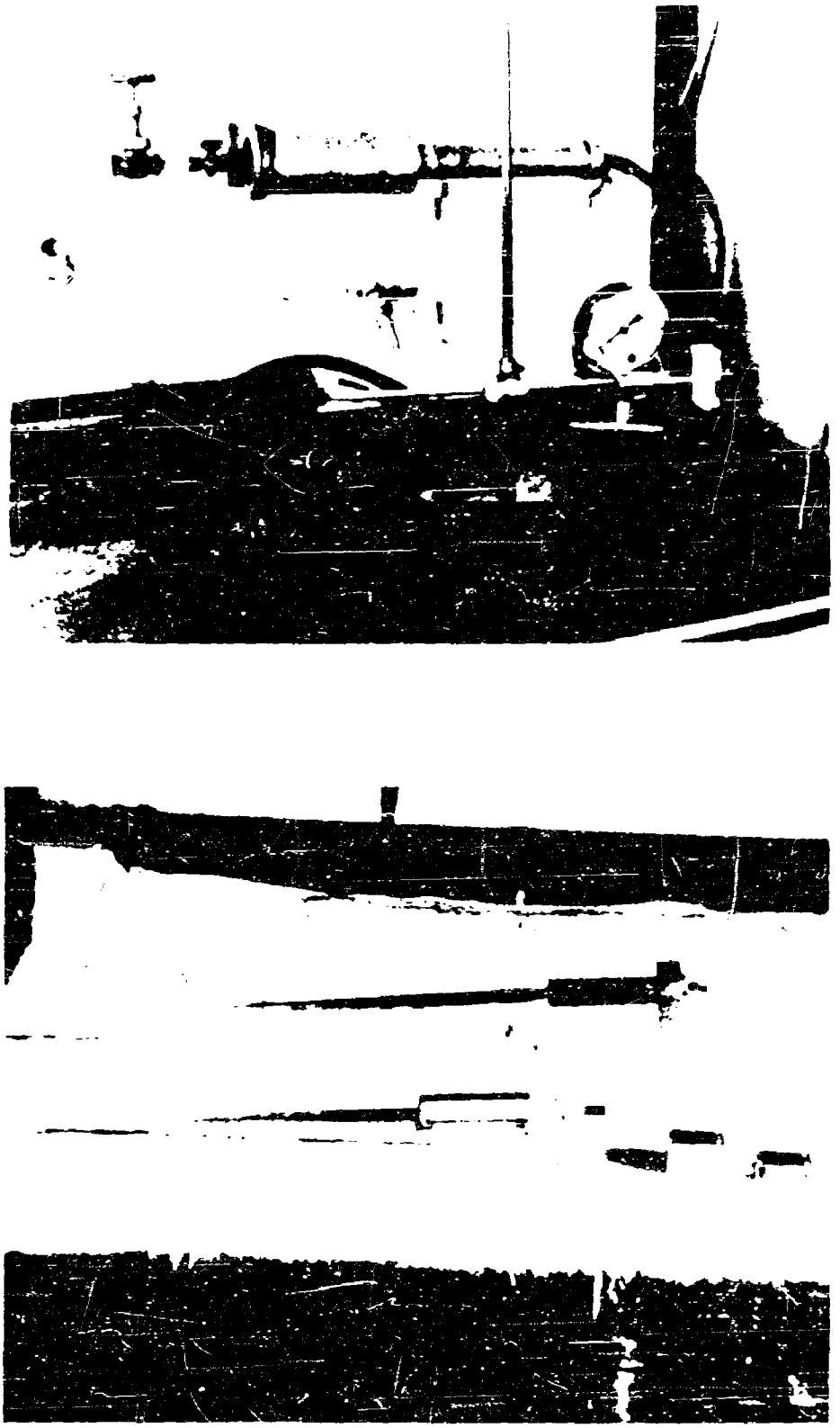


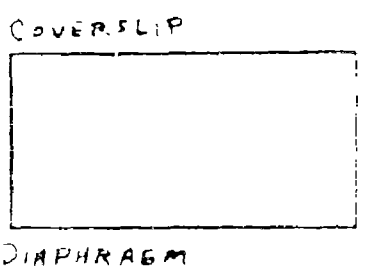
Fig. 27 Type T whistles and exposure containers.

place by rubber bands until the chamber is screwed on to the main body of the whistle. The chamber is filled with the suspension, (4 to 5 milliliters depending on the individual chamber used), and the top of the chamber is closed with a glass cover slip sealed in place with sterile vaseline. The air from the whistle maintains the exposure chamber at approximately room temperature. The increased atmospheric pressure in the resonant cavity bows the Koroseal diaphragm up, and forces some of the suspension out through the vaseline. Following exposure 1 milliliter of suspension is removed directly to a 99 milliliter water blank.

As in the siren, the sound pressure levels in the liquid are limited by those in the air below the diaphragm. In the siren the highest values obtainable were about 174 db above threshold, i.e. 0.14 at. pressure amplitude. By incorporating the cavity into the resonant generator, values as high as 0.32 at. pressure amplitude have been recorded. (In comparing these two values it should be noted that the siren data is based on rms measurements of the fundamental frequency only, whereas the whistle data are actual peak measurements.)

It was hoped that by driving the whistles at 60 to 70 psi, very stable sound fields would result. Due to personnel troubles, this assumption was not tested until after most of the bacteriological data had been taken. Fig. 28 shows a block diagram of the equipment used for measuring the sound fields in the liquid inside the whistle chamber. Distilled water was used in place of the bacteriological suspension and the cover glass was omitted. (Unlike the siren data we did not find any erratic results which could be traced to a failure to degas the water. The cover glass is so thin that it contributes very little to the sound field.) Our results are shown in Table 17.

These observations indicate that for a fixed diaphragm and with our probe in a fixed position in the liquid, the acoustic pressures were constant to about $\pm$ 10% and the frequencies to better than $\pm$ 2%. However changing diaphragms shifted the frequency by several times these limits of error, and altered not only the magnitude but also the relative importance of the various overtones. The data also clearly points out the absence of a simple relationship between the peak and absolute pressure amplitudes. The wave shape is extremely complex and difficult to analyze. Nonetheless one can approximate the sound field in the exposure chamber as the sum of two plane waves one moving up and the other down. These are described mathematically by the following equations:



$$V_u = V_0 e^{i\omega(t - h/c)} \qquad P_u = \rho c V_0 e^{i\omega(t - \frac{h}{c})}$$

$$V_d = -V_0 e^{i\omega(t + h/c)} \qquad P_d = -\rho c V_0 e^{i\omega(t + \frac{h}{c})}$$

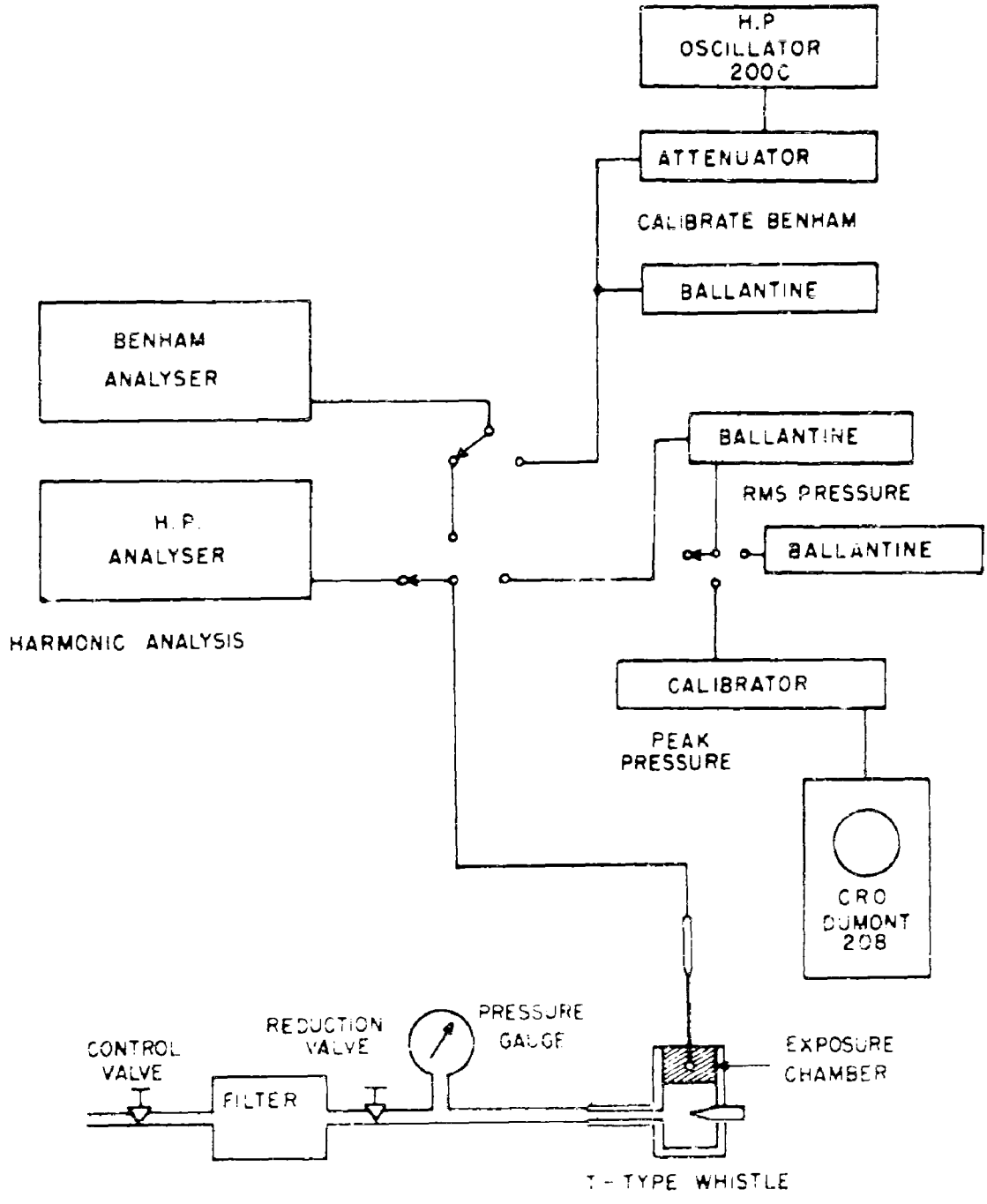


Fig. 28 Block diagram of equipment used for measuring the sound fields in the liquid inside the whistle chamber.

Table 17

Frequency in kc at position 7A

P	F_1	F_2	H_1^1	H_2^1	H_1^2	H_2^2	H_1^3	H_2^3	H_1^4	H_2^4	H_2^5
5	6.0	5.8	11.9	-	18	-	25	-	33	-	
10	6.1	6.0	12.3	11.8	21	17	28	21	32	26	32
15	6.3	6.1	12.4	12.2	22	19	26	21	32	28	32
20	6.25	6.2	12.5	12.4	22	20	23	22	32	28	33
25	6.3	6.3	12.7	12.5	22	20	23	22	32	28	33
30	6.3	6.3	12.6	12.6	21	20	24	23	33	28	-
35	6.2	6.4	12.7	12.7	20	20	23	23	32	28	-
40	6.5	6.4	12.9	12.8	20	20	24	23	33	28	-
45	6.5	6.4	12.9	12.7	20	20	24	23	33	29	-
50	6.5	6.4	13.0	12.7	20	20	24	23	34	29	25 also on Run 2
55	6.6	6.4	13.1	12.9	22	20	28	23	34	28	
60	6.6	6.4	13.1	12.9	21	20	28	23	34	28	
65	6.6	6.4	13.3	12.9	20	20	26	23	32	28	

Frequencies in kc, variable positions diaphragm 1

Pressure		65 psi					35 psi				
Pos.		F_1	H^1	H^2	H^3	H^4	F_2	H^1	H^2	H^3	H^4
A	1	6.6	13.3	19	26	32	6.5	12.7	19	21	31
	2	6.6	13.3	19	26	32	6.5	12.8	20	26	33
	3	6.7	13.4	20	24	30	6.3	12.8	20	26	33
	4	6.7	13.3	21	23	30	6.5	12.9	20	22	32
	7	6.6	13.3	20	26	32	6.2	12.7	20	23	32
B	1	6.5	13.0	21	22	32	6.3	13.1	20	23	33
	2	6.6	13.1	18	21	30	6.4	13.1	19	23	32
	3	6.6	13.1	19	21	32	6.3	12.6	19	20	31
	4	6.5	13.1	19	21	31	6.3	12.7	20	21	37
	7	6.6	13.1	19	21	32	6.4	12.7	18	20	37
C	1	6.7	13.3	19	23	31	6.4	13.4	19	22	31
	2	6.6	13.2	18	22	33	6.4	12.6	19	23	34
	3	6.5	13.0	20	23	34	6.4	12.7	20	23	33
	4	6.5	13.1	20	23	34	6.3	12.6	20	22	33
	7	6.6	13.1	20	22	32	6.3	12.6	22	23	33

For symbols see pages 62 and 63

Table 17a

Pressures in atmospheres at position 7A

P	F_1	F_2	F_3	H_1^1	H_2^1	H_3^1	H_1^2	H_2^2	H_3^2
5	.03	.02		.011			.0005		
10	.05	.06		.03	.018		.006	.009	
15	.06	.10		.04	.03		.013	.006	
20	.004	.09		.003	.02		.013	.006	
25	.09	.10		.03	.03		.03	.006	
30	.10	.13		.04	.03		.03	.006	
35	.12	.12	.08	.03	.03	.03	.04	.006	.03
40	.12	.11	.08	.04	.04	.03	.04	.016	.018
45	.14	.13	.08	.06	.04	.03	.03	.02	.03
50	.12	.14	.08	.06	.05	.04	.03	.02	.02
55	.14	.13	.09	.07	.02	.04	.03	.02	.03
60	.14	.13	.10	.05	.04	.04	.03	.02	.03
65	.11	.20	.13	.04	.06	.04	.07	.04	.03
66-67			.11			.05			.03

P	H_1^3	H_2^3	H_3^3	H_1^4	H_2^4	H_3^4	T_1	T_2	T_3	PK_1	PK_2
5	.0003			.0004			.03	.02		.05	.02
10	.0005			.0010			.04	.07		.14	.14
15	.007	.008		.010	.003		.07	.09		.14	.16
20	.009	.007		.013	.004		.09	.09		.08	.12
25	.009	.007		.03	.005		.10	.09		.05	.14
30	.0011	.006		.13	.004		.14	.10		.15	.18
35	.04	.007	.004	.07	.006	.07	.14	.14	.14	.25	.18
40	.02	.013	.004	.06	.008	.09	.14	.14	.16	.29	.16
45	.04	.016	.006	.03	.016	.11	.16	.15	.18	.29	.20
50	.03	.02	.008	.03	.011	.08	.14	.14	.16	.31	.19
55	.016	.02	.007	.013	.014	.008	.15	.14	.14	.26	.14
60	.018	.02	.008	.016	.014	.006	.13	.15	.14	.25	.17
65	.03	.09	.007	.02	.018	.03	.13	.20	.16	.26	.32
66-67			.008			.13			.20		

For symbols see page 62 and 63

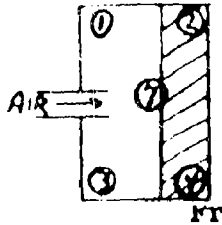
Table 17b

Pressures in atmospheres at variable positions, diaphragm 1

Pressure		65 psi							35 psi						
	Pos.	F	H1	H2	H3	H4	T	PK	F	H1	H2	H3	H4	T	PK
A	1	.12	.05	.06	.03	.018	.14	.25	.06	.03	.02	.005	.008	.11	.18
	2	.13	.05	.06	.014	.016	.12	.18	.11	.02	.011	.007	.004	.12	.18
	3	.12	.05	.09	.09	.02	.13	.28	.09	.02	.010	.007	.006	.10	.18
	4	.11	.04	.06	.06	.014	.10	.16	.06	.015	.02	.03	.009	.08	.12
	7	.11	.04	.07	.03	.02	.13	.26	.12	.03	.04	.04	.07	.14	.25
B	1	.09	.04	.018	.014	.014	.08	.08	.08	.03	.02	.010	.014	.06	.07
	2	.08	.015	.03	.03	.02	.11	.15	.07	.02	.03	.010	.008	.07	.09
	3	.09	.02	.03	.03	.013	.07	.10	.04	.010	.016	.02	.006	.04	.07
	4	.07	.03	.02	.03	.006	.05	.08	.04	.012	.03	.008	.009	.04	.08
	7	.07	.03	.06	.07	.014	.10	.19	.06	.015	.02	.02	.008	.05	.07
C	1	.004	.004	.011	.00[illegible]	.001	.018	.03	.004	.0013	.007	.003	.001	.007	.02
	2	.016	.007	.013	.010	.004	.03	.08	.013	.0018	.001	.04	.0007	.014	.02
	3	.008	.004	.010	.009	.002	.02	.02	.004	.002	.001	.001	.0006	.006	.01
	4	.004	.002	.006	.007	.002	.02	.01	.011	.002	.004	.004	.002	.009	.01
	7	.015	.007	.011	.016	.003	.02	.04	.013	.001	.008	.004	.002	.015	.02

Tabular summary of data developed from study of the jer T type whistle

Position key



A = bottom layer
B = middle layer
C = top layer

Top View

For symbols see page 63

Key to tables

P = pressure in psi of air exciting the whistle.

F = fundamental.

H = overtone.

superscripts refer to order of overtone.
subscripts refer to different diaphragms.
e.g. H_2^3 refers to the third overtone using diaphragm #2.

T = rms total acoustic pressure.

PK = "peak" pressure amplitude = 1/2 (max pressure-min pressure).

Diaphragms 2 and 3 were autoclaved.
Diaphragm 3 was used 7/24/51. Frequencies were not recorded precisely; PK was not measured.
Diaphragms 1 and 2 were used 10/1.

F and H^1 measured on Hewlitt-Packard Harmonic analyzer.

H^2, H^3, and H^4 measured on Benham analyzer.

T measured on Ballantine Electronic Voltmeter, model 300, - (Two used, first as 49 db amplifier, for very low readings).

PK measured on screen of 208 Dumont Oscilloscope using 29 or 49 db amplification (as needed) from Ballantine Electronic Voltmeter, model 300; voltages were assigned to deflections through use of Dumont 264B Voltage Calibrator.

Benham analyzer was calibrated twice an hour by comparison with a Hewlitt-Packard 200C oscillator whose output was read on a Ballantine Electronic Voltmeter, model 300, and attenuated by a Hewlitt-Packard 350B Attenuator Set.

In this: v is particle velocity; p is pressure; i is the $\sqrt{-1}$; t is time; h is height below the top of the chamber; c is the velocity of sound; and ω is 2π times the frequency of the sound wave. The subscripts u and d represent quantities associated with the upward and downward waves respectively, whereas values without subscripts refer to the total quantities. The above equations are now added giving:

$$V = V_u - V_d = V_0 e^{i\omega t}\left[e^{-i\omega h/c} + e^{i\omega h/c}\right] \approx 2V_0 e^{i\omega t}.$$

$$P = P_u + P_d = \rho c V_0 e^{i\omega t}\left[e^{-i\omega h/c} - e^{i\omega h/c}\right] \approx 2\rho V_0 e^{i\omega t}\, \omega h.$$

$$P \approx i\rho\omega h V$$

Solving these one finds:

$$|V| \approx \frac{|P|}{\rho\omega h}$$

For $|p| = 0.3$ at., one calculates $|v|$ at 6 kc as follows:

$$|V| = \frac{0.3 \times 10^6}{1.6 \times 10^3 \times 2} \doteq 4 \text{ cm/sec}$$

This is admittedly a crude calculation, but should give the correct order of magnitude.

A similar but more complex calculation for the siren indicates that peak velocities of about 1 cm/sec should have been present near the surface of the liquid.

The complex nature of the sound fields in the whistle make these extremely difficult to evaluate. The effect of the physical variations on the biological results could not be observed since no form of monitoring was carried out. Hence the bacteriological results are significant only to the extent that the average of several runs with one strain could be compared to the average of several runs with another strain. Individual variations of factors of two or more between runs are not significant.

WHISTLE SURVEY

A survey was conducted to determine the relative resistance of 13 bacterial species to the sound field of a Type T whistle. Where effects were obtained with the whistle operated at 65 psi air pressure exposures were also made at 60 psi air pressure. All

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* These approximations can be made since the height of the chamber is small compared to the wavelength of sound in water at 6 kc; expressed mathematically this means:

$$\frac{\omega}{c} h = 2\pi \frac{6 \times 10^3}{1.5 \times 10^4} \times 2 \approx \frac{2\pi}{10} < \frac{\pi}{2}$$

exposures were of 180 minutes duration.

Typical results of this survey, as tabulated in Table 18, revealed that Micrococcus pyogenes var. aureus, Strain III, and Corynebacterium michiganense were highly susceptible to the sound field of the whistle when operated at both 60 and 65 psi air pressure. Of the other five species susceptible to the sound field produced at 65 psi air pressure two, Corynebacterium xerose and Pseudomonas aeruginosa, were less susceptible to the conditions produced at 60 psi air pressure. In the case of Serratia marcescens and Sarcina lutea slight effects were observed at 65 psi air pressure while none resulted when the whistle was operated at 60 psi air pressure. However, sound pressure measurements indicate the acoustic fields to be similar for 60 and 65 psi air pressure. Very slight effects were observed with Aerobacter aerogenes at 65 psi air pressure. The other six species were resistant to the exposure conditions. Ovine erythrocytes were also exposed under these conditions but no lysis of the cells occurred on exposure of 60 min. However, this was done in the fall of 1951 several months later than most of the other experiments.

As the percentage of cells of M. pyogenes var. aureus failing to grow following exposures of 180 min was similar to that obtained in the earlier work with the siren following 90 min exposure, a time study was conducted. Due to the small volume of the container interval sampling was not possible so each exposure had to be made on a separate aliquot of the suspension and two suspensions were used. The results of these exposures are shown in Table 19.

It was found that 90 min exposure to the whistle resulted in approximately 90% of the cells failing to grow while an equal exposure to the sound field of the siren had resulted earlier in approximately 99% of the cells failing to grow.

Also it is interesting to note that slight effects were obtained with Serratia marcescens while this species had been resistant to the conditions established in the sound field of the siren. Streptococcus zymogenes and Micrococcus varians were resistant to both the whistle and the siren sound fields.

Exposures were made in October 1951 on suspensions of M. pyogenes var. aureus, Strain III, that had been degassed just prior to exposure. This was undertaken in an attempt to see whether or not the presence of gas (air) altered the effects of the sound. Because of the length of time needed to obtain significant effects it was impossible to maintain the suspension in a gas free state during the entire run.

Table 18

Exposure to the sound field of Type T whistle, operated at 60 and 65 psi air pressure, for 180 min.

Species	60 psi control cells milliliter	60 psi exposed cells milliliter	60 psi % killing	65 psi control cells milliliter	65 psi exposed cells milliliter	65 psi % killing
Micrococcus pyogenes var. aureus, Strain III	95,000,000	500,000	99	130,000,000	1,200,000	99
Corynebacterium michiganense	110,000,000	300,000	99.8	8,500,000	200,000	98
Corynebacterium xerose	16,000,000	5,600,000	65	40,000,000	2,300,000	95
Pseudomonas aeruginosa	270,000,000	160,000,000	42	44,000,000	13,000,000	71
Serratia marcescens	190,000,000	190,000,000	0	130,000,000	84,000,000	37
Sarcina lutea	140,000,000	130,000,000	0	2,300,000	1,800,000	23
Aerobacter aerogenes	-	-	-	180,000,000	150,000,000	17
Escherichia coli	-	-	-	140,000,000	130,000,000	0
Streptococcus lactis	-	-	-	11,000,000	11,000,000	0
Streptococcus zymogenes	-	-	-	24,000,000	24,000,000	0
Micrococcus varians	-	-	-	840,000	750,000	0
Lactobacillus casei	-	-	-	52,000,000	50,000,000	0
Bacillus danicus	-	-	-	9,400,000	9,500,000	0

Table 19

Percentage of cells of Micrococcus pyogenes var. aureus, Strain III, failing to grow following exposure to the sound field of the whistle operated at 65 psi air pressure for varying time intervals.*

Time in min	Exposure #1	Exposure #2
30	78	68
60	88	85
90	92	89
120	90	90

* This data was taken in June 1951 and could not be repeated in October 1951

SECTION V

CONCLUSIONS

The complex nature of the sound fields in the liquids and the lack of reproducibility from one run to the next made the physical interpretation of the data almost impossible. Moreover, it was impossible to determine if cavitation was the critical phenomenon involved. Most acoustic bacteriocide (or bacteriostasis) has been shown to depend on cavitation by observing the decreased lethal effects if the suspension was either degassed or subjected to an increased hydrostatic pressure. The latter was impossible with any of the exposure chambers used because a thin flexible coupling membrane was necessary between the air borne sound and the liquid; any distortion of this membrane by excess pressure would alter the sound field. Degassing was useless because in the lengths of time necessary to conclusively demonstrate that anything was happening to any culture now on hand (one hour or more) the suspension would have probably become completely regassed by the bacteria themselves.

The absence of suitable measuring equipment made it impossible to determine the particle velocities in the liquid. The complex character of the sound field made it difficult to determine the effect of varying the frequency and impossible to compare the siren and whistle results. Thus it is impossible to state from these experiments what physical factor, or factors, were responsible for the lethal results.

The extremely long exposures necessary with either the whistle or siren and the apparently few species affected by these fields make both of these impractical instruments for antigen or enzyme extractions. The sound fields in liquids are limited by the sound pressure in the air. Due to the relative incompressibility and the much greater density of liquids the sound pressure (0.3 at.) which corresponds to large intensities in air (100 w cm^{-2}) corresponds to negligible intensities in liquids (0.03 w cm^{-2}). Thus it is clear that high intensity air borne sound generators will never be practical for producing high intensities in liquids.

In spite of these difficulties, it is possible to draw several meaningful conclusions from these experiments. Perhaps the most striking is the fact that sound fields generated in air can be transmitted to a liquid at a sufficiently high level to produce lethal effects on bacteria. This has been accomplished without any focussing in the liquid. The sharp threshold for these effects is indicated by the Micrococcus pyogenes var. aureus studies on the siren and also by the contrast between the whistle and siren results. Although the rms acoustic pressures in the

whistle chambers were only about twice the rms values in the siren exposure tubes, Serratia marcescens which was apparently unaffected in the siren field (i.e. lethal effect less than the 25% limits of error) was 35% killed in the whistle chamber in three hours.

In the case of M. progenes var. aureus this lethal effect was accompanied by the actual breaking of the cells. This is illustrated in the photomicrographs of samples treated in the sound field. The breaking probably occurred in more than one step since longer exposures were necessary to release a given percentage of antigen than to alter the reproductive ability of the culture.

Perhaps the most interesting conclusion from the whistle data is the fact that the various species could be placed in a sequence according to their sensitivity to the sound field. All of these bacteria are much less sensitive than red blood cells to a more intense sound field, but were destroyed at significant rates in the whistle chamber where red blood cells remain unaltered. Thus one cannot help but wonder if the effects are due to the sonic field alone; no other alternative is suggested however. No correlation could be found between the physical or physiological characteristics of the bacteria and their sensitivity to the whistle fields. Nor were the observed morphological characteristics of the progeny of surviving cells altered.

SECTION VI

SUMMARY

The lethal effects of low intensity sound in liquids were studied using several species of bacteria. The acoustic fields were generated in some of the experiments by a siren, in the remainder by a Jer T type whistle. Special techniques and exposure chambers had to be developed to make these experiments possible. The experiments failed to elucidate the mode of action of the acoustic field in producing lethal effects. The data do show that bacteria in suspension can be "killed" at these low sound levels, and that some actual cellular disruption does occur. The results also indicate an extremely sharp threshold for these effects, and a relative scale of bacterial sensitivity to low intensity sound field.

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